ESTROGEN SIGNALING IN COLON INFLAMMATION AND COLORECTAL CANCER

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Stockholm 2020

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB

ISBN 978-91-7831-984-8

Estrogen signaling in colon inflammation and colorectal cancer
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The public defense at Karolinska Institutet and Royal Institute of Technology will be held at Campus Flemingsberg, Neo, Erna Möller lecture hall, floor 5, Blickagången 16, Huddinge

Friday, October 30st 2020 at 09.30

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To my brother Lukas, you will always be with me ♥

ABSTRACT

Colorectal cancer (CRC) is the third most deadly form of cancer in the Western world. Although screening efforts have reduced the overall mortality, the incidence is increasing among young adults. The frequency of inflammatory bowel disease (IBD) and obesity are increasing in parallel, which suggest a common underlying environmental link between the conditions. This increase is thought to correlate to an increased intake of high fat diets, and obesity is a major risk factor for CRC. Chronic inflammation, which is a hallmark for CRC promotion, is a well-known underlying factor in both obesity and IBD. The gut microbiota is another hallmark, and an impaired relationship between the host and gut microbes can contribute to obesity, IBD and CRC. The risk-benefit balance of current CRC-preventative treatments is poor, and there is a need for safer and better preventatives in order to reduce the CRC mortality. Both obesity and IBD place men at a significant higher risk of CRC compared to women. This indicates a protective role for estrogen. The use of full Estrogen receptor (ER) β knockout mice has demonstrated ERβ protective effects against experimentally induced CRC. However, it is unknown through which cells these protective effects are mediated. There are only low mRNA levels of ERβ in the colon, unclear if adequate for a functional role, and ERβ may also be expressed in intestinal immune cells. Understanding the CRC-preventative effects of intestinal epithelial ERβ in both sexes is important and may provide the background for a novel CRC chemopreventive approach.

The overall aim of the thesis is the functional characterization of intestinal epithelial ERβ during colon inflammation and colitis-induced CRC and identification of potential sex differences, which can ultimately provide novel opportunities for chemopreventive exploitation (Figure 1).

In **paper I** we utilized intestinal epithelial ERβ knockout mice ($ER\beta KO^{Vil}$) of both sexes and induced colitis and colitis associated CRC (CA-CRC). We found that *intestinal epithelial* ERβ is protective against colitis and CA-CRC in *both* sexes, but in a sex-dependent manner. The underlying mechanism includes an intricate crosstalk with TNFα-induced NFκB signaling.

In **paper II** we identify that both sex and intestinal epithelial ERβ impact the microbiota composition. This may contribute to the exacerbated colitis and colitis-induced tumor formation observed in $ER\beta KO^{Vil}$ mice.

In **paper III** we induced colon inflammation by feeding the mice a high-fat diet (HFD, 60%) for 13 weeks and explored treatment with estrogen receptor-selective ligands. We identified that estrogen signaling, via ERβ, modulated the HFD-induced changes in the colon microenvironment. This included sex-dependent effects on epithelial cell proliferation, macrophage infiltration, and regulation of core circadian clock gene expression.

In **paper IV** we utilized paired-normal and CRC clinical samples and identified sex differences in the transcriptome of both normal colon and CRC. By applying data-driven feature selection and machine learning on sex-separated TCGA data, we proposed sex-specific diagnostic biomarkers and prognostic biomarkers using survival analysis.

In summary, this thesis characterizes intestinal epithelial ERβ as a novel chemopreventative target for CA-CRC in both sexes, and identifies related biological pathways, including crosstalk with nuclear factor κB (NFκB) signaling and modulation of circadian clock genes. ERβ activity in intestinal epithelial cells is manifested by altered microbiota composition, cell proliferation and immune cell infiltration. The identification of several significant sex differences provides evidence for the need to take sex into account in colitis and CRC research to improve health interventions.

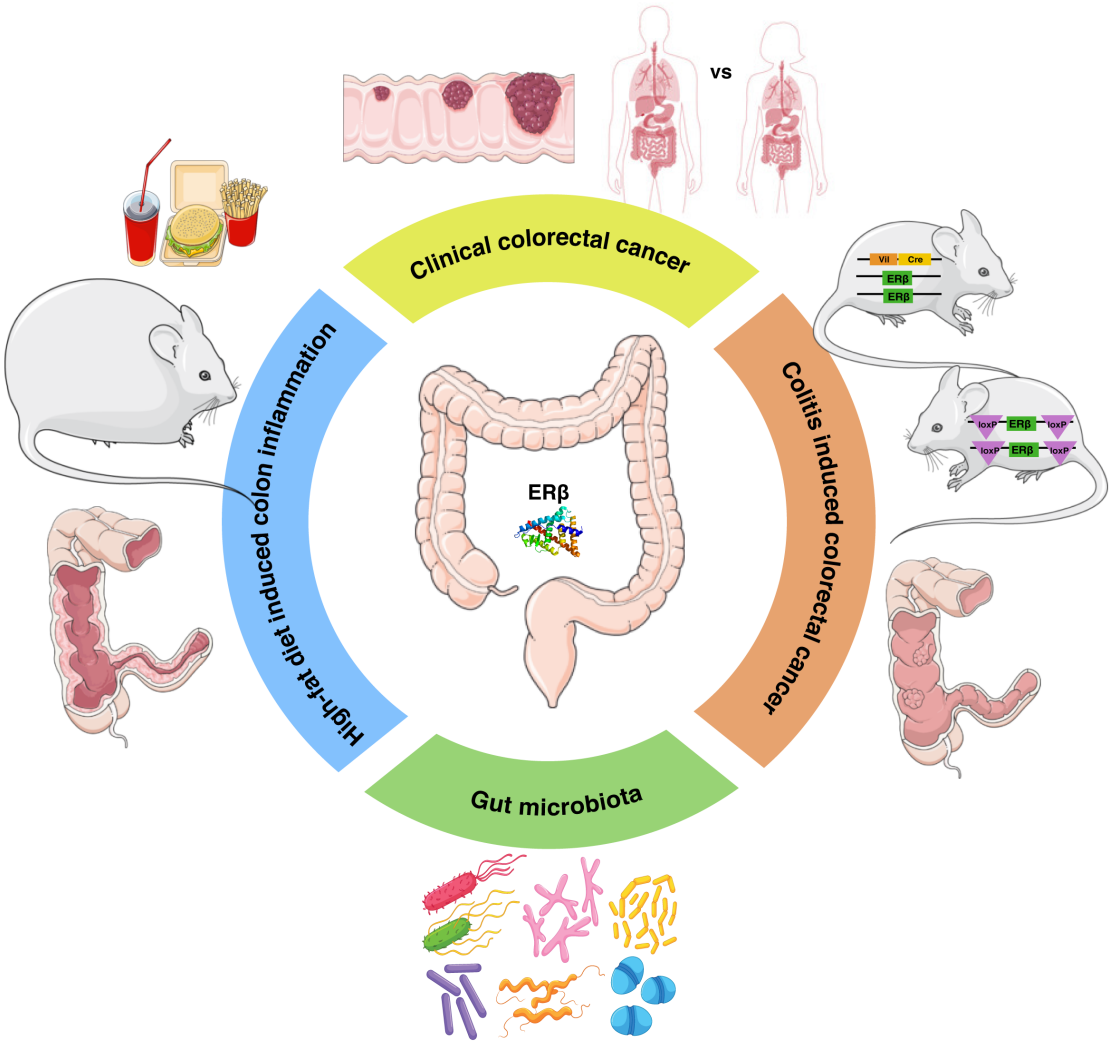

Figure 1: The aim of the thesis, built upon **paper I-IV** is to characterize the role of intestinal epithelial ERβ during colon inflammation and colitis-induced CRC, and identify potential sex differences, in order to provide novel opportunities for chemoprevention. Note: this image contains elements that were modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License and Medical vector created by brgfx - www.freepik.com.

LIST OF SCIENTIFIC PAPERS

I. **Hases L**, Indukuri R, Birgersson M, Nguyen-Vu T, Lozano R, Saxena A, Hartman, J, Frasor J, Gustafsson JÅ, Katajisto P, Archer A, Williams C. Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes. (2020) Cancer Letters. 492:54-62.

II. Ibrahim A, Hugerth LW, **Hases L**, Saxena A, Seifert M, Thomas Q, Gustafsson, JÅ, Engstrand L, Williams C. Colitis-induced colorectal cancer and intestinal epithelial estrogen receptor beta impact gut microbiota diversity. (2019) Int J Cancer. 144:3086-3098.

III. **Hases L***, Archer A*, Indukuri R, Birgersson M, Savva C, Korach-André M, Williams C. High-fat diet and estrogen impact the colon and its transcriptome in a sex-dependent manner. (2020) Scientific Reports. Online. https://doi.org/10.1038/s41598-020-73166-1

IV. **Hases L**, Ibrahim A, Birgersson M, Liu Y, Hartman J, Williams C. The importance of sex in colorectal cancer biomarker discovery. (Manuscript)

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

AdaBoost	Adaptive Boosting
AF-1/2	Activation function-1/2
AMP	Antimicrobial peptide
AOM	Azoxymethane
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
ATF3	Activating transcription factor 3
BA	Bile acid
BCL3	B-cell lymphoma 3-encoded protein
βERKO	Full-body ERβ knockout
BIRC3	Baculoviral IAP repeat containing 3
BMI	Body mass index
CA-CRC	Colitis associated colorectal cancer
cAMP	Cyclic adenosine monophosphate
CD	Control diet
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
CRC	Colorectal cancer
DBD	DNA binding domain
DC	Dendritic cell
DPN	Diarylpropionitrile
DSS	Dextran sodium sulfate
E1	Estrone
E2	17β-estradiol
E3	Estriol
E4	Estetrol
EGFR	Epidermal growth factor receptor
ERα/β	Estrogen receptor α/β
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase

FAP	Familial adenomatous polyposis
GALT	Gut associated lymphoid tissue
GPER1	G protein coupled estrogen receptor 1
H_2S	Hydrogen sulfide
HFD	High fat diet
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IgA	Immunoglobulin A
IGF1R	Insulin-like growth factor 1 receptor
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LBD	Ligand binding domain
MΦ	Macrophage
MAPK	Mitogen-activated protein kinase
M cell	Microfold cell
MCLK	Myosin light chain kinase
MCP-1/CCL2	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
MHT	Menopausal hormone therapy
MIP-1β/CCL4	Macrophage inflammatory protein-1β
MMR	Mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stable
NFκB	Nuclear factor-κB
NR	Nuclear receptor
OVX	Ovariectomized
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PPT	Propylpyrazoletriol
RF	Random forest

ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SCN	Suprachiasmatic nucleus
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SP-1	Stimulating protein-1
SRB	Sulfate reducing bacteria
TF	Transcription factor
TJ	Tight junction
TLR	Toll-like receptor
TMB	Tumor mutational burden
TNFα	Tumor necrosis factor α
TNM	Tumor-node-metastasis
WT	Wild type

1 INTRODUCTION

1.1 COLORECTAL CANCER

Cancer is the second leading cause of deaths worldwide and in 2018 it accounted for 9.6 million deaths of which 862 000 constituted of colorectal cancer (CRC) [1]. Cancer is not a single disease; it is a group of diseases described by an abnormal cell growth with the ability to spread to other parts in the body. Normal cells transform into cancer cells essentially through mutations in their genes. It is a multistage process, not a result from a single gene mutation, but rather the incorporation of several mutations. Mutations in genes controlling cell growth and survival, so called tumor suppressor genes and oncogenes might lead to uncontrolled cell growth, which can lead to cancer.

1.1.1 Etiology

CRC is the third most deadly form of cancer among both women and men in the Western world [2]. Most colorectal tumors develop via multiple steps, including a series of morphological, histological and genetic changes that slowly accumulate in the course of time. CRC typically develops from benign, precancerous polyps, which are aggregations of abnormal epithelial cells within the intestinal mucosa. The theory behind CRC development is that it arises from stem or stem-like cells that reside in the crypt bottom [3]. Cancer cells must acquire multiple mutations in order to develop into cancer. Genomic and epigenomic instability is a hallmark for CRC progression, which can lead to mutations and epigenetic changes in oncogenes and tumor suppressor genes, which facilitates the malignant transformation from adenomas to carcinomas [4].

1.1.2 Genomic instability

CRC is a heterogeneous disease with a number of genetic and epigenetic changes. Three molecular pathways leading to CRC have been identified including chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways [4]. The CIN pathway is characterized by an imbalance in chromosome number and a high degree of loss-of-heterozygosity of tumor suppressor genes [5]. MSI is characterized by defects in the mismatch repair (MMR) machinery [6]. MMR is a conserved mechanism correcting for DNA replication errors and defects in this mechanism leads to alternate sized microsatellites and subsequent frameshift mutations in tumor suppressor genes [6]. The majority of MSI-high tumors show a high tumor mutational burden (TMB) [7]. The CIMP pathway is characterized by hypermethylation of CpG islands, which leads to silencing of tumor suppressor genes [8]. One tumor can present characteristics of more than one pathway, thus the pathways are not mutually exclusive.

1.1.3 Pathophysiology

Up to date, two distinct models for the transition of normal colon epithelium to CRC have been identified (Figure 2). They both transforms from normal epithelium to aberrant crypt foci, followed by early adenomatous polyps (adenomas) and progression to CRC [9]. This process can take 10–15 years, but can progress more rapidly if there is a genetic predisposition, such as familial adenomatous polyposis (FAP) or Lynch syndrome [4]. Adenomas are benign tumors and less than 10% of adenomas develop into CRC [10]. The two morphological pathways include the classical pathway and the alternative serrated neoplasia pathway [9]. In 1990, Fearon and Vogelstein proposed the model for the adenoma to carcinoma sequence, where certain mutations are associated with different stages of CRC progression [11]. This classical pathway is the most common and has become a widely accepted standard for solid tumor progression. It is driven by CIN and involves the progression of tubular and tubulovillous adenomas, which can progress into adenocarcinomas [12]. According to this model, mutation in the tumor suppressor gene adenomatous polyposis coli (*APC)* is the first event, leading to aberrant activation of Wnt/β-catenin signaling, followed by mutation in the oncogenic *KRAS*, and deletion of chromosome 18q and inactivation of the tumor-suppressor gene *TP53* [12]. The serrated neoplasia pathway, on the other hand, is a more recent discovery. This involves the progression of serrated adenocarcinoma from serrated polyps. It arises from histological and molecular events different from tubular adenomas and is associated with mutations in the oncogene *BRAF*, with CIMP as a primary driving force and MSI as secondary driving force [13].

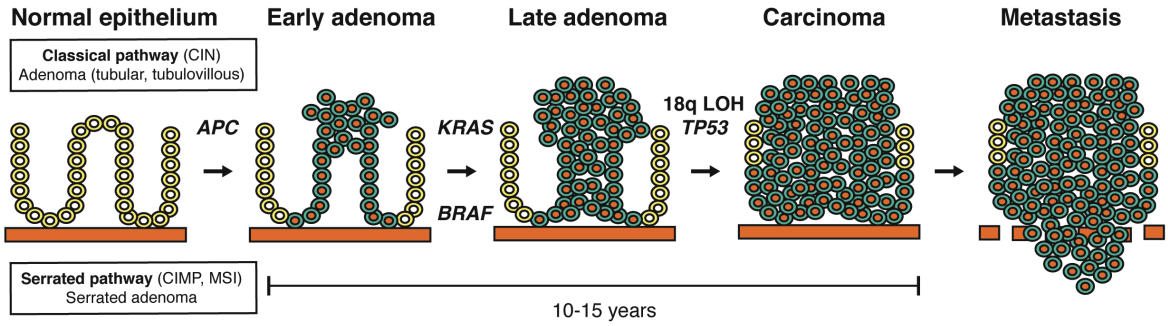

Figure 2: CRC progression. The classical pathway involves CIN and is characterized by tubular adenomas and early mutations of *APC,* followed by mutations in *KRAS* and *TP53* and LOH at chromosome 18q. The serrated pathway involves CIMP and *BRAF* mutations and MSI as secondary driving force and is characterized by serrated adenomas.

1.1.4 Tumor staging and molecular classification

CRC survival rates depend on the tumor stage at diagnosis. The tumor-node-metastasis (TNM) staging is the most common staging model used, and is based on the depth of tumor wall invasion, lymph node spread, and metastasis (Table 1) [14]. However, TNM staging alone does not perfectly predict patient response to adjuvant chemotherapy, since patients with similar histopathology can present completely different outcome based on, for example, their genetic background. CRC is a very heterogeneous disease and characterizing the molecular subtypes of individual tumors are important for prognostic and therapeutic implication [15]. Up to date, several models for different molecular subtypes have been described. Based on the

status of the main molecular defects, including CIN, CIMP, MSI, *BRAF*- and *KRAS* mutations, CRC has been divided into five molecular subtypes; type 1 (MSI-High, CIMP+, *BRAF* mutation, WT *KRAS*); type 2 (microsatellite stable (MSS) or MSI-low, CIMP+, *BRAF* mutation, WT *KRAS*); type 3 (MSS or MSI-low, CIMP$^-$, WT *BRAF*, *KRAS* mutation); type 4 (MSS or MSI-low, CIMP-, WT *BRAF,* WT *KRAS*); and type 5 (MSI-High, CIMP-, WT *BRAF,* WT *KRAS*) [16]. However, up to date, only a few genomic biomarkers are routinely used for treatment prediction in the clinic, including *KRAS* and *BRAF* mutational status and MSI status [17]. *KRAS* mutational status is a marker for predicting resistance to anti-epidermal growth factor receptor (EGFR) drugs [17]. MSI status is a predictor of immunotherapy due to a high TMB and a high load of tumor neoantigens [18]. CRC patients with MSS tumors with high TMB may also benefit from immunotherapy. In fact, FDA recently approved pembrolizumab, an immunotherapy, for the treatment of all metastasizing solid cancers with high TMB (≥10 mutations/megabases) [19].

Table 1: Staging of CRC based on TNM classification [14]. T describes the tumor size and whether it has invaded nearby tissue. Tis stands for carcinoma *in situ* and refers to a group of abnormal cells that has not spread to surrounding tissue. N describes the degree of spread to regional lymph nodes. M describes the presence of distal metastasis.

Stage	T	N	M
0	Tis	N0	M0
I	T1-2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3-4a	N1/N1c	M0
	T2-3	N2a	M0
	T1-2	N2b	M0
IIIC	T4a	N2a	M0
	T3-4a	N2b	M0
	T4b	N1-2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b
IVC	Any T	Any N	M1c

1.1.5 Tumor location

Additionally, tumor location denotes an additional source of tumor heterogeneity (Figure 3). Left-sided (distal colon) and right-sided (proximal colon) CRC differ in their molecular characteristics, clinical outcome and treatment response [18]. Right-sided CRC is more often found in women and characterized by sessile serrated adenomas whereas right-sided CRC is most often found in men and defined by traditional adenomas [20]. While right-sided CRC patients tend to have more CIMP, MSI-high and *BRAF*-mutated tumors, left-sided CRC patients are associated with CIN, *TP53* and *APC*-mutated tumors [20]. Left-sided CRC patients have a better overall survival compared to right-sided, with better response to adjuvant chemotherapies such as 5-fluorouracil and targeted therapies such as anti-EGFR therapy [18]. Right-sided CRC, respond poorly to conventional chemotherapies, but demonstrates promising response to immunotherapies [18].

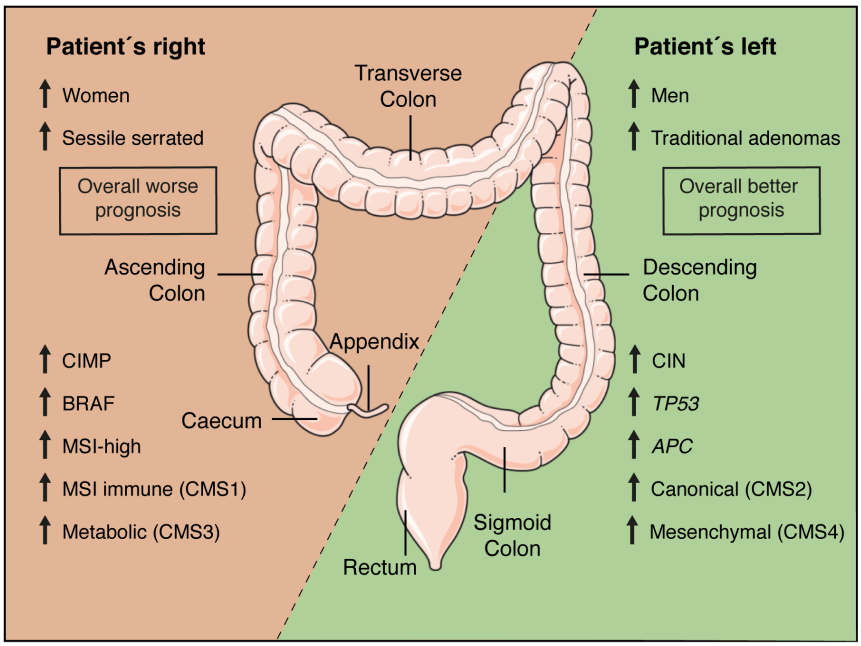

Figure 3: Characteristics of left-sided and right-sided CRC. Note: this image contains elements that were modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License.

1.1.6 Risk factors

Not only genetic factors, but also environmental factors contribute to the etiology of CRC. Several risk factors, both modifiable and non-modifiable, have been recognized. The non-modifiable risk factors include the individual susceptibility to develop CRC, and age is the most significant. The average age at diagnosis is 64 and more than 90% of the people diagnosed are older than 50 [21]. Other non-modifiable risk factors include family history and hereditary factors. Family history accounts for approximately 20-30% of patients with CRC [22]. The risk doubles in individuals with a first-degree family member diagnosed with CRC at 50-70 years of age, and triples if diagnosed before 50, and the risk further increases if there is two or more affected family members [4]. Hereditary CRC syndrome accounts for approximately 10% of all patients and the most common one is Lynch syndrome, caused by

mutations in DNA MMR genes [4]. The second most common is FAP, caused by mutations in the tumor suppressor gene *APC* [4]. Inflammatory bowel disease (IBD), leading to chronic colitis is associated with an elevated CRC risk, which increases with the extent of IBD [23]. However, the majority of CRC cases, around 70-80%, is sporadic and arises without any genetic predisposition [22]. These are linked to environmental modifiable risk factors [10, 22], such as high-fat diets (HFDs), obesity, low physical activity, alcohol consumption, and smoking [24].

1.2 COLITIS-ASSOCIATED CRC

Chronic inflammation is a hallmark of cancer promotion and tumor growth, and promotes colitis-associated cancer through the production of oxidative stress, DNA damage, and accumulation of mutations and genomic instability [25]. A variety of pro-inflammatory mediators trigger inflammatory responses, including tumor necrosis factor alpha (TNFα/TNF).

1.2.1 Inflammatory bowel disease (IBD)

IBD is a condition of chronic inflammation in the intestinal tract and the two most common subtypes of IBD are ulcerative colitis and Crohn's disease. Ulcerative colitis is described by an inflammation that emerges in the rectum and extends proximally, but is rarely found in the small intestine. Crohn's disease is described by inflammation in the entire gastrointestinal tract, with lesions primarily in the proximal colon and the small intestine. The precise etiology of IBD is unknown, however, it is multifactorial and driven by immune system disruption and/or an imbalanced interaction with the gut microbiota triggered by certain environmental factors in a genetically susceptible host [26]. IBD patients present increased paracellular permeability, with tight junction (TJ) abnormalities [27-29], mucus abnormalities [30, 31], and gut microbiota dysbiosis [32, 33]. In addition, elevated TNFα levels, both locally and systemically, have been identified in patients with IBD [34, 35].

IBD patients are at greater risk to develop colitis-associated colorectal cancer (CA-CRC), and 20-30% of the patients will develop it at some point during their life [36]. Moreover, CA-CRC presents a higher malignant potential compared to sporadic CRC [37]. The pathophysiology of CA-CRC follows the inflammation-dysplasia-carcinoma sequence compared to the traditional adenoma-carcinoma sequence seen in sporadic CRC [38]. The mutations in CA-CRC are similar to the ones found in sporadic CRC, however the timing of mutation acquisition differs between the two. In CA-CRC, *TP53* mutations/deletions are early events and *APC* mutations, the driver mutation in sporadic CRC, occur later and are less frequent in CA-CRC [38]. This indicates that it is not the *APC* mutation *per se* that drives CA-CRC, but rather the downstream activation of oncogenic pathways, such as Wnt/β-catenin.

Chemically-induced colon tumor animal models using azoxymethane (AOM)/dextran sodium sulfate (DSS) are widely used for studying CA-CRC. A single injection with the genotoxic colonic carcinogen AOM, followed by the chemical irritant DSS administrated in the drinking water, induces toxic effects on the colon epithelium and results in tumors with histological and molecular changes that closely resemble human CA-CRC. The molecular

alterations include alteration in β-catenin, increased levels of pro-inflammatory cytokines, including TNFα, and increased activity and nuclear translocation of NFκB. [39]

1.2.2 Obesity

Epidemiological studies support that a high body mass index (BMI) increases the risk to develop CRC [40-42]. Chronic inflammation is a well-established underlying factor in obesity and can promote tumor development [43]. HFD increases this risk, and the incidence of CRC among young adults are increasing due to altered life-style factors [44]. HFD feeding to mice has recently been identified as a model to study colon inflammation. Colon has been identified as the first organ to respond to HFD [45], with increased intestinal permeability, stem cell activity, inflammation, and altered gut microbiota [46-53]. In addition, HFD-induced obesity has been shown to exacerbate CA-CRC development in mice [54].

HFD feeding has also been shown to influence the circadian rhythmicity in the hypothalamus, adipose tissue, and liver, and to impair the normal eating behavior in mice [55]. However, time restricted HFD-feeding restored the circadian metabolism and helped to prevent obesity and metabolic syndrome (MetS) [56]. Hence, the circadian rhythm may be a major contributor in the pathogenesis of HFD-induced diseases. In fact, meta-analyses have revealed a correlation between night shift work, obesity, and an increased risk of CRC [57, 58].

The frequency of IBD has increased in parallel with obesity, which suggests that there is a shared environmental link between the conditions. Adipose tissue produces pro-inflammatory adipokines, which is a possible mechanism linking obesity and IBD [59]; and circulating TNFα levels has been found to be higher in obese individuals [60]. Another plausible mechanism linking the two conditions is the altered intestinal microbiota found in both obesity and IBD [59]. However, it has been shown that HFD-induced changes in the gut microbiota in mice are independent of obesity [61], which implies that diet is a major manipulator of the gut microbiota.

1.3 CIRCADIAN RHYTHMICITY

The master clock in the suprachiasmatic nucleus coordinates the mammalian physiology in a 24-h light-dark cycle. The circadian rhythm is fundamental for the sleep-wake cycle, which is orchestrated by the regulation of the hormone melatonin. The master circadian clock sends out signals to regulate gene expression in peripheral tissues [62]. However, the peripheral tissues, such as the intestine, also possess self-sustaining circadian rhythms not regulated by the light-dark cycle, but regulated by food intake [62]. The circadian rhythm in the intestine regulates a variety of biological processes, such as intestinal permeability, cell proliferation, immune homeostasis, gut microbiota, and metabolism, and its disruption can contribute to both IBD and CRC (reviewed in Voigt *et al.*) [62].

1.4 GUT MICROBIOTA

The colon is inhabited by a tremendous number of bacteria, called the gut microbiota, which presents vital benefits to the host. The most abundant phyla belong to Firmicutes and Bacteroidetes, whereas other less abundant bacteria belong to Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia [63]. The gut microbiota produces short chain fatty acids (SCFAs) by fermentation of resistant starches and dietary fibers, synthesizes vitamin B and vitamin K, and convert primary bile acids (BAs) to secondary BAs [64]. SCFAs, especially butyrate, are energy sources for intestinal epithelial cell (IEC) and modulates epithelial cell differentiation, proliferation and apoptosis [65]. Additionally, SCFAs present important immunomodulatory functions and have been shown to increase mucus production, strengthen barrier function and inhibit inflammatory responses [66]. In contrast to SCFAs, secondary BAs metabolized by the gut microbiota increase the risk for CRC, by induction of DNA damage and genomic instability [67]. The gut microbiota is responsible for keeping intestinal homeostasis, and an impaired symbiotic relationship between the host and associated microbes, known as dysbiosis can contribute to obesity, IBD and CRC [68]. Intestinal dysbiosis is proposed to be one of the leading causes of CA-CRC, by exacerbating chronic inflammation.

A proper balance between anti- and pro-inflammatory pathways is important to maintain intestinal homeostasis. A remarkable feature of the intestinal immune system is its ability to tolerate luminal microbiota, but at the same time protect intestinal mucosa from pathogenic bacteria. In fact, the intestine has its own immune system called gut-associated lymphoid tissue (GALT), which account for nearly 70% of the entire immune system [69]. The GALT consists of isolated lymphoid follicles surrounded by follicle-associated epithelium, forming the interface between the GALT and the lumen [70]. The microbiota is important to shape the immune system and germ-free mice show defects in the development of GALT, in immunoglobulin A (IgA) antibody production, and present fewer lymphoid follicles [71].

1.4.1 Intestinal homeostasis

The epithelial lining of the colon consists of a single layer of diverse IEC (i.e. absorptive colonocytes, hormone-producing enteroendocrine cells, mucus-producing goblet cells and microfold (M) cells) [72], which are held together by adherent junctions and TJs and organized in crypts. The different cell types are generated from intestinal stem cells, located at the crypt base, whose progeny proliferate and differentiate into mature epithelial cells as they migrate up towards the luminal surface [72]. The intestinal stem cells are protected from microbiota-derived metabolites through the crypt structure, since the surface colonocytes absorb and reduce the levels of different metabolites before they reach the stem cells [73]. The IEC together with the mucus layer generates a physical barrier, which protects the intestinal mucosa from pathogens in the intestinal lumen. This separation is crucial for a healthy relationship between the gut microbiota and the host. In addition, IEC mediates a crosstalk between the gut microbiota and intestinal immune cells by secretion of cytokines, chemokines and immunomodulatory molecules to coordinate immune responses and maintain intestinal

homeostasis (Figure 4). This crosstalk is mediated by expression of pattern-recognition receptors, including toll-like receptors (TLR), that respond to microbial products [72]. TLRs are important to maintain tolerance to commensals but at the same time inducing inflammation against pathogens. The TLR functions are dependent on its subtype and cellular location. Activation of some TLR subtypes in the apical side of IEC is involved in maintaining a healthy intestinal barrier by secreting anti-inflammatory cytokines, enhancing antimicrobial peptide (AMP) production, TJ integrity, mucus secretion, and IEC proliferation and differentiation [74, 75]. In addition, pathogen infiltration activates TLRs located on the basolateral side of the IEC and immune cells in the lamina propria, which induces secretion of inflammatory cytokines to activate the immune response [74]. Activated pro-inflammatory macrophages (MΦ) are recruited to clear the bacterial infection and secrete cytokines and chemokines to recruit other immune cells, including neutrophils [76]. The resident MΦ, on the other hand, persist during the inflammation and maintain their anti-inflammatory phenotype by secreting interleukin (IL)-10, which is crucial for the mucosal healing [76]. A number of studies have shown that TLR-deficient mice are more susceptible to experimental-induced colitis and present increased intestinal permeability, bacterial burden, and inflammatory-response [77-79]. This emphasizes the gut microbiota as an important modulator of intestinal homeostasis.

IEC also direct the adaptive immune response towards gut microbes, illustrated in figure 4, by transporting luminal antigens and bacteria, through M cells, to antigen-presenting cells (i.e. dendritic cells) in the lymphoid follicles, referred to as the immune sensors of the colon [75]. Antigen presentation to B and T cells in the lymphoid follicles contributes to antigen-specific IgA production and T-cell response [75], leading to self-tolerance or immunological defense. IgA blocks bacteria and toxins from attaching to the epithelial cells, preventing colonization, epithelial damage and subsequent massive invasion [80].

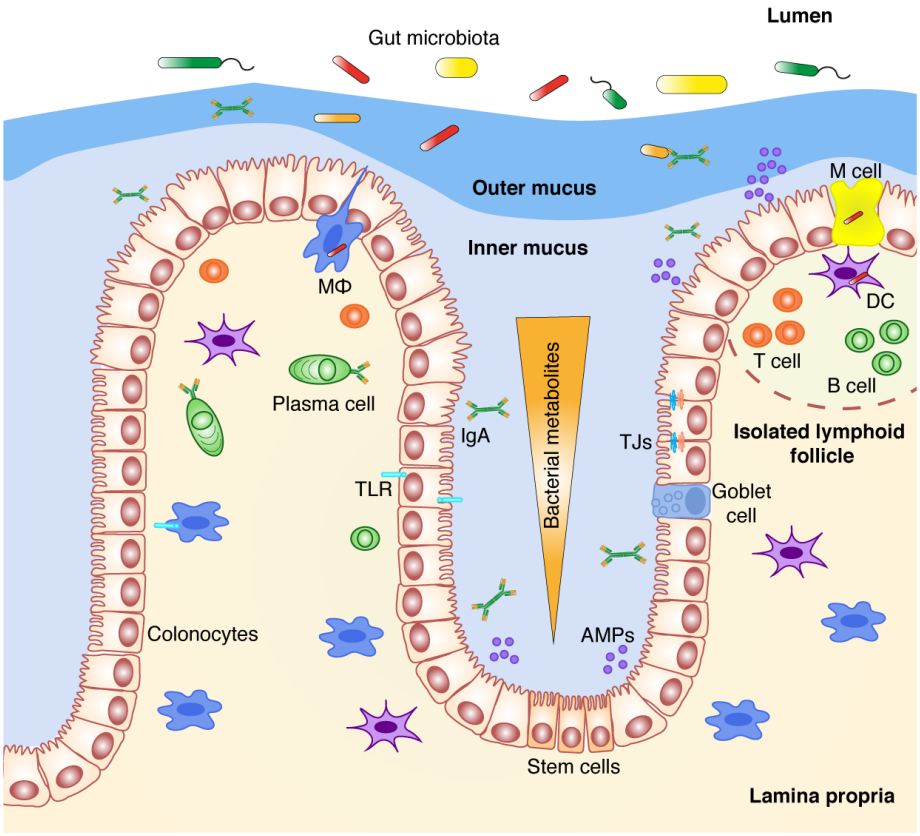

Figure 4: Intestinal homeostasis. Microfold (M) cells sample bacteria from the lumen to antigen-presenting cells, i.e. dendritic cells (DC), in the lymphoid follicles. Antigen presentation to B and T cells leads to antigen-specific IgA production and T-cell response, leading to self-tolerance.

1.4.2 Gut dysbiosis

Gut dysbiosis is characterized by loss of beneficial bacteria, outgrowth of harmful pathogens, and loss of overall bacterial diversity. This can be caused by dietary changes, inflammation, infection or exposure to toxins or antibiotics [81]. Generally, dysbiosis in obesity, IBD, and CRC is associated with a decrease of SCFA/butyrate-producing bacteria, with subsequent reduced levels of SCFAs [82], which can lead to enhanced intestinal permeability and inflammation. SCFA supplementation is protective against AOM/DSS-induced CA-CRC [83]. Reduced intake of dietary fibers, which are the main energy source for intestinal microbiota, can lead to outgrowth of bacteria that can utilize alternative energy sources, like mucin-degrading species. This can result in a disrupted mucosal barrier, increased bacterial translocation, and inflammation [84]. Intake of HFD increases the levels of toxic secondary BAs, which exerts detrimental effect on the colon epithelium by inducing genotoxic effects, inflammation and proliferation [85]. In addition, HFD increases the proportion of gram-negative bacteria, which correlates to increased levels of plasma lipopolysaccharide (LPS), an endotoxin that causes metabolic endotoxemia and eventually obesity-related disorders [52]. LPS binding to TLR4 activates NFκB, which leads to increased expression of pro-inflammatory cytokines and results in increased intestinal permeability [52]. The increase of reactive oxygen species (ROS) during inflammation disrupts the anaerobic environment in the gut, which can lead to dysbiosis by creating a selective advantage and overgrowth of facultative anaerobes or some aerobes [86]. Intake of HFD has been shown to

increase the ratio of Firmicutes to Bacteroidetes [51]. In addition, dysbiosis induces an excess growth of potential harmful bacteria in the gut, like the pathogen *Enterobacteriaceae*, belonging to the phylum Proteobacteria, which is one of the most commonly overgrown pathogens seen in IBD, obesity, and CRC [87]. By secreting enterotoxins it induces intestinal permeability and damage to IEC, leading to intestinal inflammation [88]. The altered microbiota metabolites, through release of toxic metabolites, seen in dysbiosis may be associated with neoplastic changes in IEC.

1.5 TNFA/NFKB SIGNALING

TNFα plays a critical role in inflammation in both obesity and IBD, and is a target for biological treatments. TNFα is an early-response cytokine that controls several cellular processes, such as apoptosis, differentiation and cell proliferation, and production of inflammatory molecules, which are all linked to response to epithelial cell injury [89]. TNFα act as a promoter of inflammation-associated cancer by activating the pro-survival transcription factor (TF) NFκB [90]. NFκB is highly activated during many inflammatory conditions, and is a critical driving force of inflammation-induced cancer, by inducing several oncogenes and cytokines involved in carcinogenesis [91-93]. Intestinal epithelial activation of NFκB leads to inflammation-induced CRC [94].

The NFκB TF family consists of five subunits: c-Rel (REL), p65/NFKB3 (RELA, NFKB3), RelB (RELB), p105/p50 (NFKB1), and p100/p52 (NFKB2), that form transcriptionally active homo- and heterodimeric complexes. In unstimulated conditions, NFκB dimers are sequestered in the cytoplasm by interacting with IκB proteins. TNFα activates the canonical NFκB pathway, leading to activation of IκB kinase (IKK) complex, which phosphorylates the IκB proteins. The IκB proteins are then degraded, which allows nuclear translocation of the NFκB and subsequent transcription of its target genes [95]. TNFα activation of NFκB increases, for example, the expression of myosin light chain kinase (MLCK), which increases the intestinal permeability [96]. In addition, TNFα acts as a potent mutagen causing DNA damage via the production of ROS [97].

Anti-inflammatory drugs are the most common treatment for patients with IBD, which also prevent the development of inflammation-induced CRC. Anti-inflammatory treatment with aspirin reduces the incidence of CRC in clinical trials [98, 99], by inhibiting NFκB nuclear translocation [94]. However, the risk-benefit balance of using anti-inflammatory drugs, such as aspirin, remains poor and better preventive approaches are needed. In addition to aspirin, anti-TNFα therapy is also used to treat patients with IBD. However, 30-40% of patients do not respond to this treatment, and an additional 30-40% may loose the response over time [100]. TNFα can enhance epithelial wound healing, and several mouse studies have shown that depending on the inflammatory context, TNFα can also suppress intestinal inflammation. Reduced TNFα levels by antibody treatment or knockout in a mouse model of acute DSS-induced colitis aggravated the severity of colitis [101]. However, in contrast to the findings for acute colitis, reduced TNFα levels attenuated chronic DSS-induced inflammation [102 103]. These studies indicate a beneficial role of TNFα during acute colitis but a detrimental effect

during chronic colitis. Several side effects of anti-TNFα treatment in IBD patients have been noted, including elevated risk of bacterial infection [104] and neurological complications [105]. The risk-benefit balance of current therapies to treat patients with IBD remains poor and there is a need to develop better and safer preventatives to reduce the prevalence of CA-CRC.

1.6 ESTROGEN SIGNALING

1.6.1 Estrogen hormones

Estrogens are a class of cholesterol-derived sex steroid hormones important for the function and development of female and male reproductive organs, and numerous other functions. In premenopausal women, estrogens are synthesized mainly in the ovary. Ovarian theca cells synthesize androgen, which is aromatized to estrogens by aromatase (CYP19A1) in neighboring granulosa cells [106]. In males, estrogens are primarily synthesized in Leydig cells of the testis, where aromatase is expressed [106]. Furthermore, aromatase is highly expressed in the placenta, and also expressed at lower levels in extra-gonadal sites, such as adipose tissue, muscles and brain, where estrogens also can be synthesized [106]. There are three main forms of circulating endogenous estrogens (in order of potency): 17β-estradiol (E2), estrone (E1) and estriol (E3). E2 is the predominant circulating estrogen in premenopausal women [107]. In men and postmenopausal women, the circulating estrogen levels are low, with E1 being predominant, mainly produced by aromatization of androgens in the adipose tissue [107]. E3 is the predominant circulating estrogen during pregnancy, which is produced in large quantities by the placenta [107]. An additional estrogen, estetrol (E4) is a weak estrogen also found only during pregnancy. It is exclusively synthesized in the fetal liver and enters the maternal circulation via the placenta [108]. There are also minor endogenous estrogens, which do not require aromatase for their synthesis, such as the androgen metabolite 3β-diol, synthesized from dihydrotestosterone (DHT). Also, 3β-diol is important for the male reproductive system [109]. The schematic illustration of estrogen synthesis is visualized in figure 5.

In addition to its critical role for the reproductive organs, estrogens play fundamental roles in various physiological functions. It is responsible for maintaining a normal function of the cardiovascular system [110], bone metabolism [111], energy metabolism [112], immune response [113-115], and central nervous system [116]. At the same time, estrogen signaling dysfunctions are implicated in numerous pathological conditions, including MetS [117, 118], autoimmune diseases [119], cancers of the reproductive tissues, [120-122] and some cancers of the non-reproductive tissues, e.g. CRC [123]. Estrogen, through regulation of cytokine production, can present both pro-inflammatory and anti-inflammatory properties, depending on the tissue context and dose [124]. The estrogen actions are mediated via estrogen receptors (ERs).

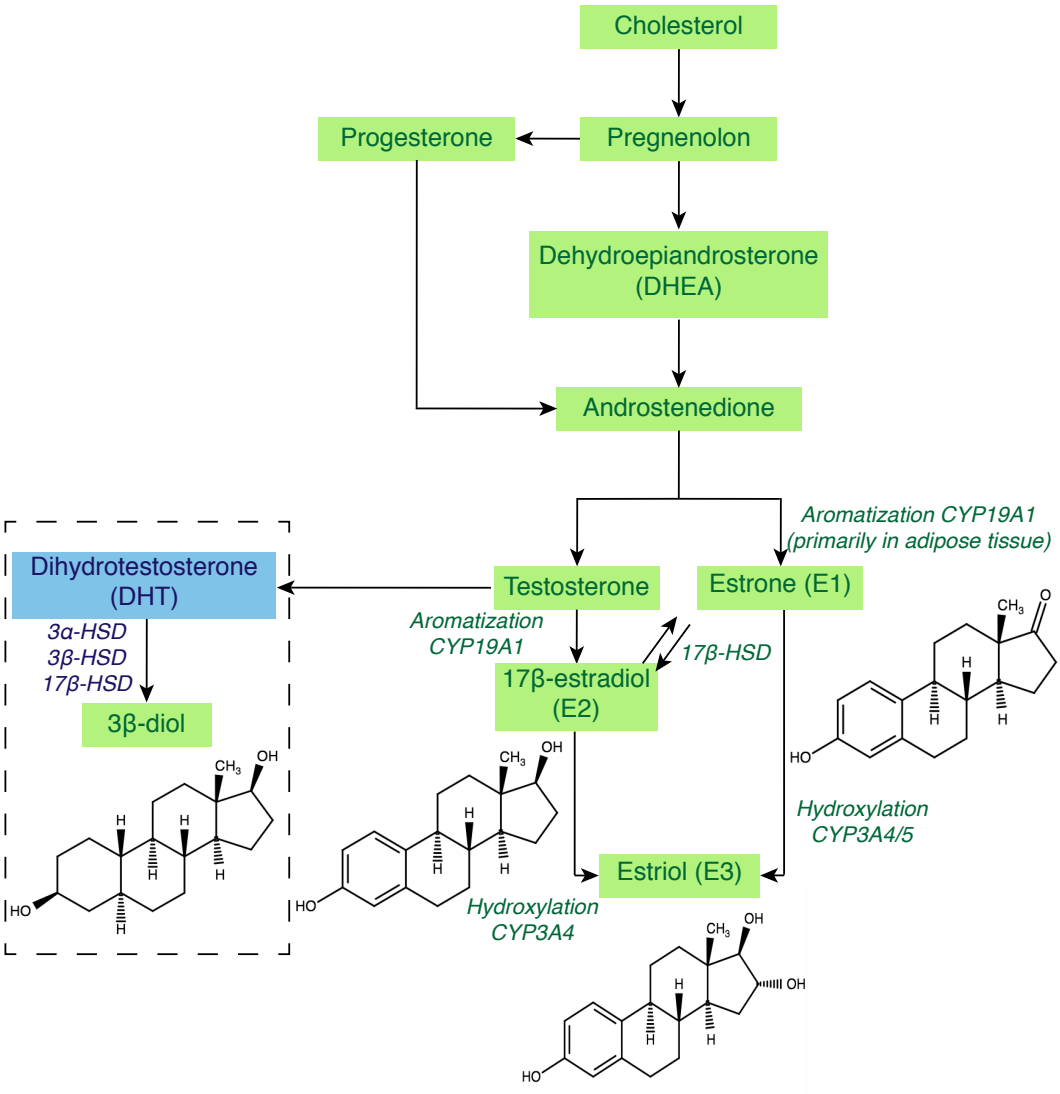

Figure 5: Schematic illustration of estrogen synthesis, including the androgen metabolite 3β-diol.

1.6.2 Estrogen receptors

Estrogen mediates its effect via ERα, ERβ and the transmembrane G protein coupled estrogen receptor 1 (GPER1). ERα and ERβ belong to the nuclear receptor (NR) superfamily, which are a family of ligand-regulated TFs. Once activated, the nuclear ERs directly regulate gene transcription to control various biological processes, including inflammation, apoptosis, cell proliferation, metabolism, and development [125]. Although the nuclear ERs mainly function as TFs, they have also been found to regulate different cellular functions in the cytoplasm [126].

There are today 48 human NRs identified, which control numerous biological and pathological processes. They are ideal targets for drug discovery, and can be activated or inactivated by small lipophilic molecules, which can easily be substituted by a drug. Thirteen percent of the FDA-approved drugs target NRs [127].

Although the NRs have different functions and gene targets, they all share similar structures [128-130]. They consist of a variable N-terminal (domain A/B), a DNA-binding domain (DBD, or C domain), a hinge region (D domain), a ligand-binding domain (LBD or E domain) and a variable C-terminal (F domain) (Figure 6A). The LBD and DBD are highly conserved between all NRs and are linked together with the hinge region. The N-terminal on the other hand is highly variable and also harbor transcriptional activation functions, so-called activation function-1 (AF-1), which is ligand-independent. The LBD contains the activation function (AF-2), which is dependent on ligand binding and plays important roles for NR homo- and heterodimerization [131]. In addition, NR binding to coactivators and corepressors is often mediated through one of its activation domains [130].

ERα, encoded by the ESR1 gene located on chromosome 6, was discovered in 1958 [132]. The second ER, ERβ, encoded by the ESR2 gene located on chromosome 14 was discovered and cloned years later in 1996 [133]. ERα and ERβ are homologues, with highly similar DNA-binding domains (97%) but variable ligand-binding and activation domains [130].

ERs are evolutionary conserved between species, including human and mouse [134]. However, splice variants of both the human and mouse ESR2 gene have been identified, which are species specific. Evidence suggests that there are five different ERβ isoforms in humans (ERβ1-5), which results from alternative splicing of exon 8, the last coding exon (Figure 6B) [135]. Structural analysis reveals that ERβ1, also referred to as ERβ wild type (WT), is the only full-length functional isoform with an intact LBD [135-137]. Two major splice variants have been identified in mice, including ERβ1 (WT ERβ) and ERβ2 (ERβ_ins). The latter contains an 18-amino acid insert in the LBD between exon 5 and 6 (Figure 6C) [134, 138].

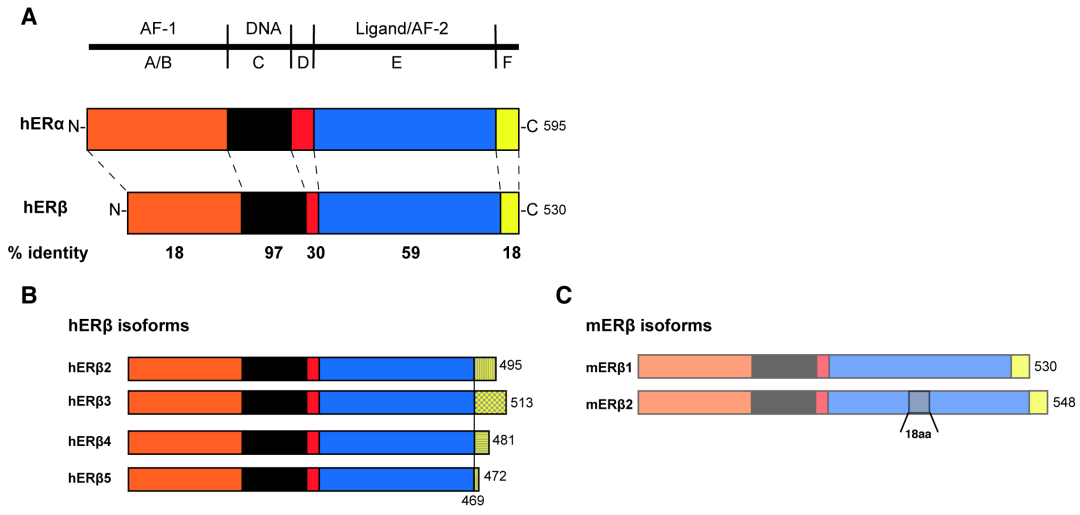

Figure 6: The structure of ERs. A) The ERs consist of five domains; A/B domain (N-terminal, ligand independent), C domain (DNA binding domain), D domain (hinge region), E domain (ligand binding domain) and F domain (C-terminal). The two ERs, ERα and ERβ share 97% sequence similarity in their DNA binding domain but variable ligand binding and activation domains. **B)** Five human ERβ isoform and **C)** two mouse isoforms have been characterized.

1.6.3 ER signaling pathways

The ER-dependent signaling can be initiated in the nucleus or in the plasma membrane [107], and divided into genomic and non-genomic signaling, based on whether the signaling regulates gene transcription. Estrogen activates ERs, but ERs can have activities also without ligands. Further, although the majority of estrogen signaling is mediated via ERs, ER-independent signaling, where estrogen regulates enzymatic activities through other mechanisms, have also been observed (Figure 7) [107].

1.6.3.1 Nuclear ER genomic signaling

The main signaling pathway is mediated by ligand activation of the nuclear ERs, which leads to genomic effects (transcriptional regulation). Binding of ER ligands to the LBD of the receptors results in a conformational change, leading to dimerization of the receptors followed by DNA binding to the estrogen response elements (EREs) [139]. Binding to EREs on the DNA leads to recruitment of co-activators or corepressors, which activates or suppresses gene transcription [139]. Apart from acting directly through EREs to regulate the expression from ERE-containing promoters or enhancers, ERs can regulate gene transcription through tethering to other TFs, such as stimulating protein-1 (SP-1), activator protein-1 (AP-1) and NFκB [107]. In addition to estrogen-dependent signaling, ligand-independent gene regulation has also been observed, where the ERs are activated through phosphorylation, by e.g. EGFR and IGF1R [107].

1.6.3.2 Membrane ER signaling

In addition to the genomic effects mediated by nuclear ER activation, some estrogen responses have been shown to be too rapid to be mediated by target gene transcription. Non-genomic signaling is common for steroid hormones, including estrogens, and are most often associated with the membrane bound GPER1. However, ERα has also been found in the plasma membrane. The nuclear ERs do not contain a transmembrane domain and the ability of ERα to be localized to the plasma membrane is dependent on interaction with caveolin-1 (CAV1), which is necessary for ERα transportation to caveolae rafts in the plasma membrane [140]. Another plausible mechanism may be due to palmitoylation of the receptor [140]. The rapid signaling by membrane bound ERs regulate a variety of different cytoplasmic effects, including mobilization of intracellular calcium, cyclic adenosine monophosphate (cAMP) production, activation of membrane tyrosine kinase receptors (e.g. EGFR and insulin-like growth factor 1 receptor, IGF1R) and protein kinase signaling cascades [107, 139]. The activation of protein kinase signaling cascades, such as cAMP/Protein kinase A (PKA), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), Phosphoinositide 3-kinase (PI3K)/AKT can also result in indirect gene regulation by phosphorylation of TFs [107, 139]. Membrane ERα signal transduction through P13K and ERK has been noted in mouse liver by the use of different ERα transgenic mouse models. The signal transduction through P13K and ERK were similar in WT mice and mice exclusively expressing the membrane ERα in mouse liver, and completely lost in full ERα knockout mice [141].

1.6.3.3 ER-independent signaling

Altough the majority of estrogen signaling is mediated via ERs, estrogen can interact with enzymatic activities in the cytoplasm to exert antioxidant activities in an ER-independent manner. Estrogens antioxidant activities are mediated by its phenolic A ring, which allow it to regulate redox activities. Studies have shown that estrogen can reduce oxidative stress by preventing ROS release by damaged mitochondria [142].

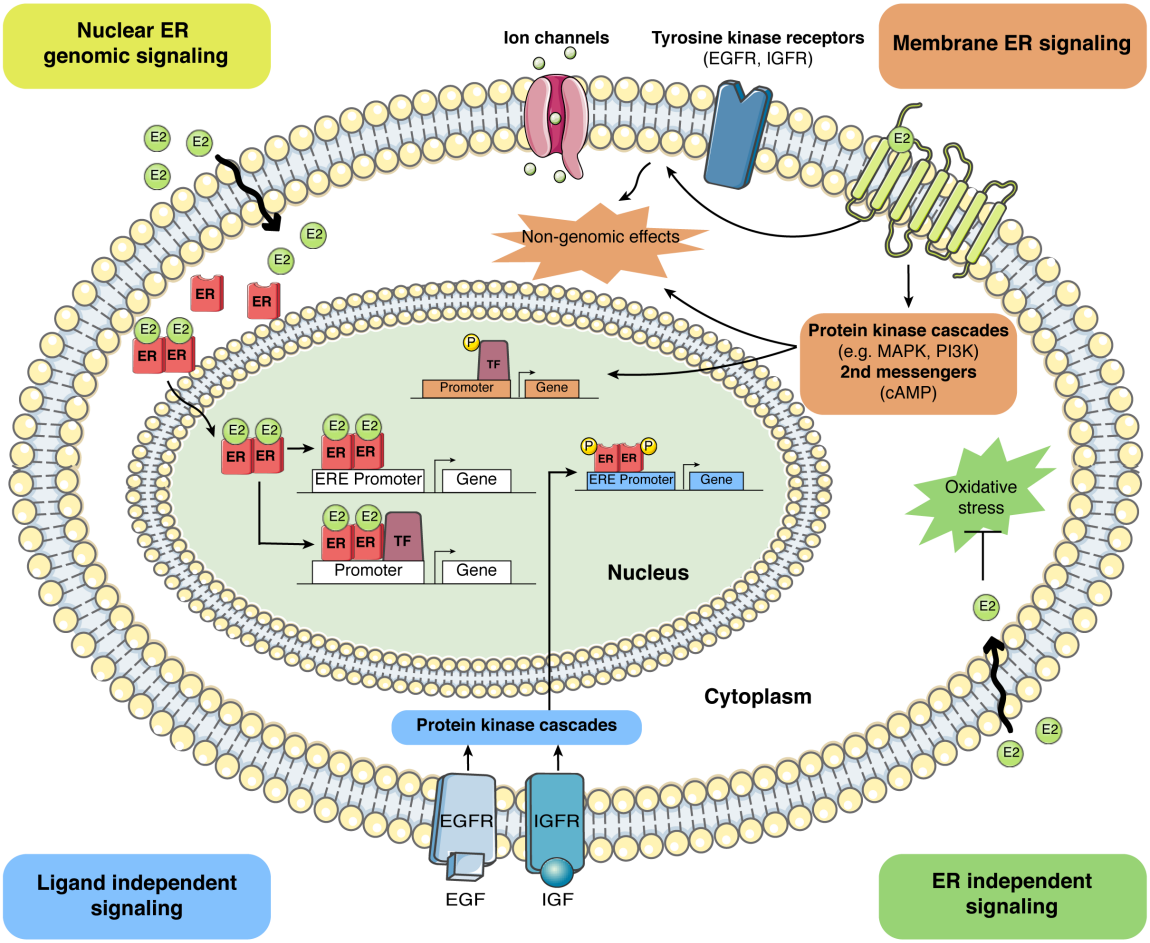

Figure 7: Estrogen and ER-mediated signaling pathways. There are four estrogen- and/or and ER-mediated signaling pathways. The first pathway is the estrogen-dependent nuclear ER signaling that mediates transcription of estrogen-responsive genes with or without EREs. The second pathway is the estrogen-dependent membrane signaling, which initiates cytoplasmic effects by regulating membrane-based ion channels, tyrosine kinase receptors, regulating protein kinase cascades, and modification of transcription factors. The third pathway is the-ER independent signaling, where estrogen exhibit antioxidant effects. The fourth pathway is the ligand-independent ER signaling, where growth factors activate protein kinase cascades, which leads to phosphorylation of nuclear ERs. Note: this image contains elements that were modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License.

1.6.4 Ligands

Endogenous estrogens activate the ERs, but many other compounds structurally similar to estrogen, referred to as xenoestrogens, can also activate ERs by binding to the LBD. These can be found in the environment and include natural phytoestrogens and synthetic estrogens [143]. Plant-derived phytoestrogens, such as the isoflavone genistein found in soy, have affinity for both ERs [144]. Certain synthetic estrogens can be found in pesticides and plastics and can, when released into the environment as pollutants, interfere with the endocrine signaling, and are thus called endocrine disruptors [143].

Based on their properties, ligands are divided into partial or full agonists, antagonists, and selective estrogen receptor modulators (SERMs). Agonists activate the ERs upon binding, whereas antagonists block the activation. SERMs are synthetic compounds, which modulate the action of ERs dependent on the tissue context. This provides the opportunity to selectively activate or inhibit estrogen actions in different tissues [143]. Tamoxifen is a commonly used SERM, which possess anti-estrogenic activities in breast cancer and is used to treat ER+ breast cancer patients [145]. At the same time it possess estrogen-like effects in other tissues, such as bone, where it protects against osteoporosis [145]. Tamoxifen binds to both ERα and ERβ [146]. In addition there is a group of antagonists referred to as selective estrogen receptor degraders (SERDs), which only presents anti-estrogenic effects. Fulvestrant is a well-known SERD, and by binding to ERs it inhibits E2 from binding, leading to a conformational change that impairs receptor dimerization [147]. The fulvestrant-ER complexes are unstable and leads to ER degradation [147]. Fulvestrant is used as a therapeutic strategy for advanced ER+ breast cancer [148].

Estrogenic ligands can bind with equal affinity to the two nuclear ERs (e.g. E2), or present higher binding affinity for one of them due to their difference in LBD (59% homology, Figure 6A). This allows for selective modulation of estrogen action via ERα, ERβ, or both. The ERα-selective agonist Propylpyrazoletriol (PPT) and the ERβ-selective agonist Diarylpropionitrile (DPN) were utilized in the studies included in this thesis. PPT present a 410-fold greater binding affinity for ERα compared to ERβ [149], while DPN has a 70-fold greater binding affinity for ERβ compared to ERα [150].

Although ERα and ERβ share 97% similarity in their DBD (Figure 6A) and have similar activation mechanism, they exhibit different, sometimes opposing activities. For example, in breast cancer, ERα drives cellular proliferation [122], whereas in colon cells, ERβ is primarily anti-proliferative [151, 152]. Their difference (18% homology, Figure 6A) in the activation domain, responsible for recruiting co-regulatory proteins, may contribute to such differences.

Thus, selective activation of ERβ may be an ideal therapeutic candidate for CRC prevention that circumvents the adverse hormonal effects.

1.7 ESTROGEN AND CRC

1.7.1 Epidemiological studies

Several epidemiological studies support that estrogen is protective against CRC. Men have a higher incidence an earlier onset of CRC [153], and men with IBD have 60% higher risk of developing CRC compared to women [154]. In addition, premenopausal women have a better overall survival compared to age-matched men and the reverse is seen in postmenopausal women [155]. Furthermore, obesity is a stronger risk factor for CRC in men compared to women. A meta-analysis of 10 studies conducted by Frezza *et al*. showed that obesity placed men at a significantly higher risk of CRC compared to women [156], which indicates a protective role of estrogen in this context. Moreover, menopausal hormone therapy (MHT), oral contraceptives, endogenous estrogens and phytoestrogens lower the incidence of CRC [157-161]. MHT in women, has been reported to reduce CRC incidence with 20% [159, 162, 163], where estrogen alone renders the largest protective effect [164]. Additionally, several epidemiological studies indicate that estrogen is protective against obesity and MetS. Menopausal transition is associated with significant weight gain, obesity, and increased prevalence of MetS, while MHT in women with diabetes has been proven to reduce obesity, insulin resistance, and fasting glucose levels [117, 118].

1.7.2 ERβ and CRC

MetS is a risk factor for CRC. In addition to epidemiological studies, animal studies suggest that females are protected against HFD-induced MetS and that estrogen treatment improves the condition [165-168]. Moreover, males are more susceptible to AOM/DSS induced tumor formation [169, 170] and estrogen treatment in males reduced the severity of colitis and subsequent tumor formation [170]. While both ERα and ERβ have been shown to be anti-obesogenic, ERα has a clear role in improving the MetS, whereas the role of ERβ in this context is more debated. Loss of ERα leads to obesity, insulin resistance and impaired glucose intolerance in mice [171, 172]. Although studies have shown protective effects of ERβ against HFD-induced MetS [173-175], Foryst-Ludwig *et al*. reported that ERβ indeed protects against HFD-induced obesity but also caused pro-diabetogenic effects, as full-body ERβ knockout (βERKO) mice presented improved glucose tolerance and insulin sensitivity [176].

Most of the estrogen-mediated CRC protective effects, however, have been linked to ERβ. ERβ is expressed at low levels in normal IEC, declines to non-detectable levels during the progression of CRC [152], and polymorphism in the ERβ promoter region correlates with CRC risk and survival [177, 178]. Moreover, several *in vivo* studies support that estrogen through ERβ mediates its CRC-protective effects. Female βERKO mice presented increased AOM/DSS-induced tumorigenesis [179], and selective activation of ERβ in the APC^{Min} model of intestinal tumorigenesis reduces colonic proliferation and tumor formation in both sexes [180]. In addition, genistein supplementation, a phytoestrogen found in natural soy, reduced DSS-induced colonic inflammation and permeability [181]. Interestingly, genistein binds ERβ with a 30-fold higher affinity compared to ERα [182]. In line with this, ERβ has been suggested to

regulate the intestinal barrier since βERKO mice presents disrupted cell architecture and cell-to-cell junctions [183]. Anti-proliferative and anti-tumorigenic effects of ERβ have also been presented in human CRC cell lines and in xenografts [184]. However, to date, it is unknown through which cells ERβ mediates its protective effect, since ERβ may also be expressed in intestinal immune cells. The role of intestinal epithelial ERβ during colitis-induced tumorigenesis has not been studied, and characterizing this mechanism is fundamental because selective activation of ERβ may provide novel opportunities for CRC chemoprevention.

1.7.3 Estrogen and microbiota

Recent advances have suggested a synergistic action between the gut microbiota and estrogens to impact obesity and cancer. Some bacterial species can metabolize estrogen-like compounds by secretion of β-glucuronidase, which deconjugates estrogen into its biologically active forms [185]. Soy isoflavones, such as genistein, is metabolized by the gut microbiota to compounds structurally similar to estrogen that can activate the ERs [185]. In fact, the gut microbiota is one of the principle regulators of circulating estrogen, and the microbiota that is capable of metabolizing estrogens are referred to the estrobolome [185]. Dysbiosis can lead to an impairment of deconjugation, which can impact the circulating levels of estrogens.

The soy isoflavone genistein possesses antibacterial activities [144]. Studies have shown that genistein supplementation can alter the gut microbiota composition in postmenopausal women by inducing the growth of the beneficial bacteria *Bifidobacterium*, which is involved in soy fermentation [186, 187]. A meta-analysis conducted by Fang *et al.* showed that dietary supplementation with phytoestrogens in postmenopausal women significantly reduced insulin resistance [188]. Dietary intake of phytoestrogens is also associated with reduced CRC risk [189].

In addition to phytoestrogens, sex hormones can regulate the gut microbiota. A study by Zhao *et al.* showed that postmenopausal women have a lower species richness and diversity than premenopausal women [190]. Moreover, several human and animal studies support that sex impact the gut microbiota although the results are inconsistent (reviewed in Kim *et al.* 2019) [191], and the sex differences are more evident in patients with an enteric infection [192]. Inconsistent results can be due to differences between mouse strains and housing conditions. Independent analysis of the gut microbiome from 89 different inbred mouse strains revealed that sex had an impact on the microbiota composition [193]. The sex differences were indeed mediated by sex hormones since gonadectomized mice presented an altered gut microbiota under HFD-feeding in both sexes [193]. In addition, the α-diversity was significantly different between the sexes only after puberty [194]. Another study by Kaliannan *et al.* showed that intact females present significantly lower abundance of *Proteobacteria*, lower Firmicutes to Bactereoidetes ratio, higher abundance of *Akkermansia* and higher *Bifidobacterium/Enterobacteriaceae* ratio compared to ovariectomized (OVX) females and intact males fed a Western diet [195]. Furthermore, the role of estrogen is supported since E2 treatment reduced the levels of *Proteobacteria* in both males and OVX females and increased

the *Bifidobacterium/Enterobacteriaceae* ratio in OVX females [195]. Moreover, LPS-related functional pathways were lower in females, E2-treated males and E2-treated OVX females, compared to intact males and OVX females [195]. E2 treatment was also shown to impact the gut microbiota diversity in males with AOM/DSS induced CA-CRC [196].

The impact of ERs to modulate the gut microbiota has not been well studied. However, one study using female βERKO mice, showed that ERβ impacts the gut microbiota composition in a diet-specific manner [197]. Hence, it is possible that intestinal epithelial ERβ may modify the gut microbiota during colitis and CA-CRC and thereby impact the disease progression.

1.7.4 Estrogen crosstalk with NFκB

NFκB is highly active during colitis, and serve as a critical link between inflammation and CA-CRC [94]. There is a lot of evidence that estrogen exerts anti-inflammatory activities, and ERs are known to interact with NFκB. E2 via ERα has been shown to block LPS induced nuclear translocation of p65 in the RAW 264.7 MΦ cell line [198]. Moreover, E2-activated ERα and TNFα-activated NFκB have been shown to interact in both a repressive and a synergistic manner to regulate gene expression involved in cell proliferation and invasion and metastatic processes in MCF-7 breast cancer cells [199]. In addition, TNFα has been shown to strongly modify the ERα enhancer landscape in a NFκB-dependent manner [200]. Based on the homology between ERα and ERβ, and the finding that ERα can modify inflammatory responses through a crosstalk with NFκB in breast cancer, it is possible that ERβ may modify inflammatory signaling in the colon and thereby reduce the risk of cancer development. Interestingly, βERKO mice showed increased colonic activation of NFκB and iNOS production [179], and E2 treatment in males [170] and OVX females [201] inhibited the NFκB signaling pathway in AOM/DSS treated mice. In addition, NFκB has been shown to repress the ligand-dependent transactivation of endogenous ERβ in granulosa tumor cell lines [202].

2 AIMS OF THE THESIS

The overall aim of the thesis was to characterize the role of ERβ as a preventative target for inflammation and inflammation-induced CRC. An additional aim was to explore sex differences in the colon and CRC transcriptome.

In order to achieve these aims, four studies were conducted, which will be referred to as paper I-IV throughout the thesis. The specific objectives are as follows:

Paper I. Investigate the role of intestinal ERβ on inflammatory signaling and epithelial cell proliferation during colitis-induced (AOM/DSS) tumor formation in mice of both sexes, and to characterize potential sex differences.

Paper II. Investigate the impact of colitis-induced tumor formation (AOM/DSS) and intestinal ERβ on the gut microbiota composition in mice of both sexes, and to dissect possible sex differences.

Paper III. Investigate the role of estrogen signaling on the colon microenvironment during HFD-induced colon inflammation and to dissect potential sex differences.

Paper IV. Identify sex differences in the colon transcriptome in normal and paired CRC clinical samples and identify potential sex-specific biomarkers.

3 MATERIAL AND METHODS

3.1 ANIMAL EXPERIMENTS

Ethical permission for all animal work in this thesis has been approved by the Animal Care and Use Committee and local ethical committee of the Swedish National Board of Animal Research. Mice lacking ERβ specifically in the intestinal epithelial cells (referred to as ERβKO^{Vil}) and two models to induce colon inflammation and/or tumor formation were used in order to characterize the role of intestinal epithelial ERβ on the colon and the colon microenvironment during colon inflammation and colitis-induced tumor formation. In **paper I-II** we utilized a CA-CRC mouse model and in **paper III** we utilized a mouse model of diet-induced obesity. Using mouse models for studying human diseases have limitations, but mice are biologically very similar to humans and develop many of the same diseases. Critically, animal studies enable gene deletions and controlled dietary and genetic conditions, which is a necessity for this type of research.

3.1.1 Mice

ERβKO^{Vil} were generated using the Cre/lox system. Mice with the ERβ exon 3 flanked by two loxP sites, ERβ$^{flox/flox}$, were crossed with transgenic mice bearing cre recombinase expressed under the control of the enterocyte-specific Villin promoter. Cre is thus only expressed in the intestine where it performs site-specific deletion of the DNA sequence between the loxP sites. The deletion of ERβ exon 3 leads to a frameshift mutation and an early stop codon, with no resulting protein expression of ERβ. Littermates, ERβ$^{flox/flox}$ lacking the Cre allele, were used as controls (referred to as WT). The mice were on a C57BL/6J background, and the genotype was confirmed with standard PCR protocol. The advantage of using ERβKO^{Vil} mice is the ability to characterize the role of ERβ specifically in the intestinal epithelium. Of note is that ERβ is also lost in the small intestine. Other male and female C57BL/6J mice were obtained from in-house breeding.

3.1.2 AOM/DSS-induced colitis and tumor formation

Five to 10-week-old ERβKO^{Vil} and WT mice of both sexes were injected once (intraperitoneal) at day one on the first week with 10mg/kg body weight (BW) of the colonic carcinogen AOM, followed by 2.5% of the colon irritant DSS in the drinking water for the second week. This was followed by two weeks of normal drinking water, before repeating the DSS cycle, three times in total. During the DSS cycles, the mice loose weight, get diarrhea, and rectal bleeding. The mice recover during the normal drinking water and eventually develop colitis and adenomas after three cycles. The mice were sacrificed after 9 or 15-16 weeks after AOM injection. The AOM/DSS model closely resembles the pathological and molecular changes seen in human CA-CRC, and is a commonly used, highly reproducible method that can be used on mice with different genetic background. Another advantage with the DSS method is that different stages of the disease, both acute and chronic inflammation, can be studied depending on the concentration and frequency of DSS administration. One

limitation is the high AOM carcinogenic dose given to the mouse, which differs from the human situation where tumors usually develop from low exposure to carcinogens present in the environment during a longer time.

3.1.3 High-fat diet (HFD)-induced colon inflammation

Five to six-week-old male and female C57BL/6J mice were fed a HFD (60% kcal fat) or a matched low-fat (10% kcal fat) control diet (CD) for a total of 13 weeks. For the treatment with estrogenic ligands, the mice were injected every other day (intraperitoneal) for a total of 9 injections for three weeks prior to sacrifice, with 0.05 or 0.5 mg/kg BW E2, 2.5 mg/kg BW PPT, 5 mg/kg BW DPN, or vehicle. The ligands were prepared in a solution of 40% PEG400, 5% DMSO and 55% water. Compared to the AOM/DSS model, the HFD-model better resembles the human situation. However the diet used in this study does not accurately reflect the Western diet. An additional limitation with the HFD model is the common use of improper CDs that does not account for the fiber content. In this study, we used a matched CD with the same amount of fibers. Thus, our findings will accurately reflect the difference in the fat and carbohydrate content.

3.1.4 Tissue collection

Colons were harvested, washed, measured, opened along the long axis, and the number and size of the intestinal adenomas were recorded. The colons were then transferred to cryo-embedding media (i.e. OCT), or fixed in 4% formaldehyde for 24h, stored in 70% EtOH and embedded in paraffin for histological analyses and immunohistochemistry (IHC), or snap frozen in liquid nitrogen for quantitative reverse-transcription PCR (qPCR) analysis. The sections were stained with hematoxylin and eosin (H&E) for colitis scoring by a pathologist blinded to the mouse genotype. Severity, degree of hyperplasia, degree of ulceration and percentage of the area involved were scored (0-3) generating colitis scores ranging from 0-12.

3.2 ELISA

Blood was collected at sacrifice by cardiac puncture using EDTA-treated tubes. Plasma was collected by centrifugation for measurement of E2 and insulin levels. The E2 assay is based on a competitive ELISA whereas the insulin assay is based on a sandwich ELISA. For E2 measurements, anti-E2 antibody-coated wells are incubated with samples and E2 conjugated with horseradish peroxidase (HRP). During incubation, the HRP-labeled E2 competes with the endogenous E2 in the samples and the amount of the fixed HRP-labeled E2 decreases as the levels of endogenous estrogen increases. For insulin measurements, the ELISA is based on a capture antibody and a detection antibody. The insulin is first captured by the pre-coated wells with anti-insulin antibody and then detected by a biotinylated anti-insulin antibody. HRP is then added and forms a complex with the biotinylated antibody. A substrate is then added to the wells, which results in the development of a color and the absorbance is measured spectrophotometrically. As the absorbance is directly or inversely proportional to the amount of captured insulin and E2 in the samples, the amount can be derived from standard curves obtained from standards with known concentrations.

There is one limitation with measuring E2, without including E1. Circulating estrogen has been reported to increase with obesity and adipose tissue is the primary source of extra-gonadal estrogen. However, the estrogen produced by aromatization of androgens in the adipose tissue is primarily E1 . E1 is also the predominant source of estrogen in men and postmenopausal women.

3.3 RNA *IN SITU* HYBRIDIZATION

ERβ expression in clinical samples was verified in the human protein atlas database, by immunohistochemistry (IHC) with the validated mouse monoclonal ERβ antibody (PPZ0506). The expression of ERβ is relative low; hence the mouse monoclonal antibody is not optional for detecting ERβ expression in mouse, since the secondary anti-mouse IgG antibody will detect endogenous mouse IgG antibodies, leading to a high background staining. For that reason we used RNA *in situ* hybridization (ISH) for the localization of *Esr2* mRNA in the colon tissue. This hybridization uses labeled complementary RNA to localize the mRNA sequence in the tissue.

3.4 IMMUNOHISTOCHEMISTRY (IHC)

IHC is a widely used antibody-based method to visualize antigens in a cell. We used the standard indirect Avidin-Biotin Complex (ABC) method to visualize the proliferative marker Ki-67 and the macrophage marker F4/80. First, an unlabeled primary specific antibody binds to the target antigen, followed by binding of the secondary species-specific biotinylated antibody to the primary antibody. The third layer involves the formation of a complex of avidin-biotin HRP, which can catalyze the oxidation of DAB to form a brown colorimetric product that can be visualized in light microscopy.

In this thesis we used two different types of samples preparations: formalin fixed and paraffin embedded (FFPE) and frozen sections embedded in OCT. Both of these methods present advantages and disadvantages over one another. FFPE is better when it comes to storage and for morphological analyzes, since it preserves the tissue architecture by cross-linking. The conformations of proteins can be different in FFPE from frozen sections, where the proteins are preserved in its native stage, which affect recognition by antibodies. The frozen method is quicker and does not require antigen retrieval for reversing the cross-links to expose antigens. For the AOM/DSS animals we used FFPE, since we wanted to analyze the morphology of the tissue and score it for colitis. For the HFD animals we utilized the frozen method, to avoid the problem with epitope masking for some of the antibodies we used.

Before incubation with the primary antibody, the tissue needs to be blocked for unspecific staining, including endogenous peroxidase activity, endogenous biotin and for reactive epitopes. The blocking improves specific binding by the antibody. However, not all antibodies are specific for the epitope of interest. Thus, it is important to validate the antibodies and to include proper positive and negative controls. For these experiments, however, we used standard monoclonal antibodies that were the most cited. We verified that the staining pattern agreed with known expression, i.e. a confined F4/80^{+} staining in immune

cells between the intestinal crypts and a confined Ki67 staining in the nucleus of epithelial cells in the lower part of the crypts.

3.5 *EX VIVO* EXPERIMENTS

Small intestinal organoids were cultured from WT and ERβKO^{Vil} mice of both sexes to study how ERβ affect TNFα induced epithelial cell damage in isolated tissue, independent of the microbiota and immune cells. An organoid is defined as a miniature organ grown *in vitro*. The intestinal organoids were produced from stem cell-containing crypts and results in a three-dimensional structure of IEC, which resembles the *in vivo* organization with all different IEC types present when supplied with suitable exogenous growth factors and basement membrane scaffolding, in this case Matrigel. Collaborators performed this experiment. Primary organoids were cultured for 5-7 days, and treated with TNFα and/or DPN or equal amount of vehicle (DMSO), and number of *de novo* crypt domains per organoid was quantified. Quantification was conducted with coded sample identities and blind scoring. The disadvantage with this method is that we utilized small intestinal organoids instead of colonic organoids, and cannot draw strong conclusions how ERβ impacts regenerative growth and differentiation capacity in the colon. Although small intestinal cancer is rare in comparison to CRC, few studies have utilized colonic organoids, which may be due to the fact that colonic organoids are difficult to culture and maintain.

3.6 *IN VITRO* EXPERIMENTS

In order to elucidate the mechanism behind ERβ protective effects we utilized human CRC cell lines with or without (mock) lentiviral transduced full-length ERβ expression. One major limitation with using cell lines when investigating complex diseases, such as CRC, is the loss of tissue context and the influence of the tumor microenvironment. Another limitation is the study of ectopically expressed ERβ, which may not resemble the reality. However, cell lines are ideal for pure mechanistic studies, and comparing our mouse *in vivo* findings to our human CRC cell lines findings can validate molecular mechanisms.

3.6.1 Cell culture

CRC cell lines SW480 and HT29 mock and ERβ-expressing cells were cultured in normal cell culture media containing 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Phenol red in the cell culture media is a weak estrogen and FBS contains low levels of estrogen, which can interfere with the response of exogenous estrogenic treatments. Therefore, the medium was changed to phenol red-free media with 5% dextran-coated charcoal (DCC)-treated FBS 24h before experiments, still including 1% penicillin-streptomycin. DCC is used to reduce the levels of estrogen in FBS. The cells were treated with 10 nM E2, 10 nM DPN, 10 ng/ml TNFα, or vehicle for 24 h before luciferase assays. Cells were treated with E2 for 2h before ChIP experiments and treated with 10 ng/ml TNFα for 0, 30min or 2h before p65 WB experiments.

3.6.2 Luciferase assay

The luciferase assay was used to determine if ERβ and NFκB impacted each other's transactivation. A luciferase assay can be used to determine the ability of a protein to regulate gene transcription, but does not establish whether it is through DNA binding or through interaction with other protein complexes that can affect transcription. The estrogen response element (ERE) and NFκB response element (NFκB-RE) are placed upstream of the luciferase gene (LUC). Here we utilized the dual reporter assay and transfected the cells with ERE-TATA-LUC or NFκB-RE-LUC reporter plasmids, with renilla luciferase control reporter plasmid as internal control. The expression of luciferase is measured in order to quantify the activity of the regulatory element. By adding luciferase assay reagent, firefly luciferase luminescence is generated and can be quantified. This reaction is then quenched and the renilla luciferase can be measured by adding another reagent to the same tube. The results are presented as firefly to renilla luciferase activity.

3.6.3 WB p65 nuclear translocation

TNFα activates the canonical NFκB pathway, leading to nuclear translocation of the NFκB heterodimer p65/p50 allowing transcription of its target genes. Here we separated the nuclear and cytoplasmic fractions to investigate if ERβ impacts the nuclear translocation of p65. The antibodies used were towards p65, α-tubulin (for cytoplasmic fraction) and SUN1 (for nuclear fraction). The cell lysates were homogenized and the cytoplasmic and nuclear fractions were separated through sucrose cushion centrifugation, which gives rise to a cushioning effect and maintains the integrity of the nuclei. The nuclear lysate was washed and centrifuged to obtain the nuclear protein fraction. The nuclear and cytoplasmic protein fractions were loaded and separated by SDS-PAGE. The ionic detergent SDS denatures and binds to protein to give them an equal negative charge. When current is applied, the negatively SDS-bound proteins will migrate to the positive electrode and the proteins will be separated based on size as they migrate through the polyacrylamide gel. The proteins separated by size were transferred onto a PVDF membrane and blocked for unspecific protein binding with milk. The primary antibody is first added to the membrane, which binds the protein of interest, and a secondary antibody conjugated with HRP is added for detection of the primary antibody. Enhanced chemiluminescence (ECL) substrate was added and oxidized by HRP and the chemiluminescent signal was quantified. As this is an antibody-based method, non-specific protein detection is possible, due to similar epitopes. The α-tubulin and SUN1 antibodies were among the top-cited antibodies and are well characterized. α-tubulin is a monoclonal antibody, whereas SUN1 is a polyclonal antibody. Polyclonal antibodies are a mix of antibodies that recognizes different epitopes of the same antigen, which increases the risk for non-specific binding. However, we could validate our α-tubulin and SUN1 antibodies as they were only detected in the intended fractions. The p65 monoclonal antibody we used had only a few citations, but its specificity in WB has previously been validated by the use of p65 knockout.

3.6.4 Chromatin immunoprecipitation (ChIP)

Unlike the transactivation assay, ChIP can be used to investigate protein and DNA interactions in a cell. By the use of ERβ ChIP-qPCR we could determine whether ERβ binds or is localized to specific genomic regions, such as promoter regions or other DNA-binding sites. DNA binding or associated proteins are first cross-linked to the DNA using formaldehyde. The chromatin is isolated and sonicated into short fragments before incubation with ERβ antibody or IgG antibody (control) attached to magnetic beads for immunoprecipitation. The immunoprecipitated ERβ-DNA cross-links are reversed followed by purification of the DNA. Primers for qPCR were designed to align to predicted ERβ-binding sites, and negative control primer set was designed for a chromosome area where ERβ is not predicted to bind. Amplification of ERβ target sites were normalized to input from the same ChIP experiment (three independent experiments) and to Ct values of the negative controls and presented as fold enrichment. This is again an antibody-based method and it is important to have a validated antibody in order to produce reliable results. Here we use two negative controls, including the IgG ChIP and the ERβ ChIP performed in the Mock cells lacking ERβ to normalize our results, which will increase the signal-to-noise ratio. Moreover, the ERβ antibody used (PPZ0506) has previously been validated for ChIP in the lab (Indukuri *et al*., submitted) and is highly specific.

3.7 CLINICAL SAMPLES

Clinical samples (colorectal tumors and matched noncancerous adjacent tissue) were collected from patients (n=24, 18 women and 6 men) undergoing surgery in Stockholm. The study was approved by the regional ethical review board in Stockholm. In addition, gene expression for 641 (299 women and 342 men) CRC (COAD and READ) and 51 (28 women and 23 men) noncancerous mucosal tissues were downloaded from TCGA. The data were downloaded on 31st of January 2019 with the bioconductor package (31) from R via NCI Genomic Data Commons (GDC) data portal.

3.8 QUANTATIVE PCR (QPCR)

Frozen mouse tissues and clinical paired normal and CRC tissues stored in RNAlater were homogenized with a tissue lyser. RNA was isolated and purified and DNAse treatment was used to remove any genomic DNA. RNA was reverse transcribed to cDNA. Stool pellets from mice were homogenized and DNA was extracted. The cDNA or DNA was detected using qPCR. In qPCR, the amplification of the target sequence is monitored in real time and we used a fluorescent dye called SYBR Green, which binds to double-stranded DNA. Fluorescence can be quantified at the end of each amplification cycle to determine the amount of DNA that has been amplified. The cycle threshold value (Ct) is the number of cycles where the fluorescent signal is significantly greater than the background fluorescence. The Ct value is typically detected where the product amplification is exponential and the amount of product is doubled for each cycle. The Ct value is inversely proportional to the amount of target nucleotide sequence present in the sample. The Ct values are used to calculate the relative fold gene expression using the ΔΔCt method. The Ct value for each

sample is first normalized to a reference gene (i.e. ΔCt), which should be stable between samples and conditions. Next, the ΔCt difference between two experimental groups (ΔΔCt) is calculated. The fold change in the expression of the gene of interest between the experimental groups is then equal to $2^{-\Delta\Delta Ct}$.

3.9 OMICS TECHNOLOGIES

Omics technologies were applied in all four papers, in **paper I, III, IV** we used transcriptomics to study how sex and ERβ impact gene expression and in **paper II** we used 16S rRNA-sequencing to study how sex and ERβ impact the gut microbiota community.

In **paper I**, we utilized Illumina bead array for the transcriptomic analysis of two human CRC cell lines with and without ERβ expression and treated with TNFα (2h) to study how ERβ impact the TNFα-induced CRC transcriptome. In **paper III** and **IV** we used RNA-sequencing with Illumina NovaSeq6000. In **paper III** we investigated how HFD, sex and estrogen signaling impacts the colon transcriptome and in **paper IV** we investigate deregulated genes in CRC compared to normal colon and study sex differences in the normal colon and CRC transcriptome.

3.9.1 Illumina bead array

RNA was prepared from triplicates of SW480 and HT29 CRC cell lines with and without ERβ expression and with and without 2h TNFα treatment. RNA was converted to labeled cRNA used for hybridization onto the Illumina Whole-Genome Gene Expression Direct Hybridization Array, with 25,559 probes included for analysis. After hybridization a scanner reads the signal and the relative expression levels for each gene are received by comparing the signals between different conditions. Lumi and limma packages were used to determine differentially expressed genes. Genes were considered as differentially expressed if P-value<0.05 and log2FC>|0.4|. Gene ontology/biological function and subnetwork enrichment analysis were performed in Elsevier's Pathway Studio (11.2.5.9).

3.9.2 Illumina RNA-sequencing

RNA from mouse colon tissue was prepared from 6 biological replicates from male and female fed with CD and HFD, and males fed with HFD and treated with E2 (0.5mg/kg BW). RNA from clinical samples (paired normal and CRC tumors) was prepared from 24 patients undergoing surgery in Stockholm. Library preparation was done with TruSeq Stranded Total RNA library preparation and rRNA was depleted with Illumina RiboZero. With this method, RNA is converted to cDNA, fragmented, and adapters are ligated to the end of the fragments. Single-stranded cDNA will hybridize to the flow cell through their adapter sequence. Once attached, cluster generation begins, which makes thousands of copies of each DNA fragment through bridge amplification, which will emit a signal strong enough for detection when sequenced. After cluster generation, all reverse strands are washed away leaving several million of dense clusters with identical sequences, and the sequencing can begin. A primer is attached to the forward strand and fluorescently labeled nucleotides are added and the first

labeled base is incorporated into the new DNA strand. Only one nucleotide can be incorporated at each run and a laser record the fluorescence after each run, and each base give rise to a different color. The process continues and the DNA sequence is analyzed base-by-base. At least 17M paired-end reads (2x51bp in length) were generated for each sample. The reads were mapped against the mouse genome (GRCm38) or the human genome (GRCh37) using STAR. FeatureCounts and StringTie were used to generate gene counts, TPM and FPKM values. DESeq2 was used to calculate differentially expressed genes (DEG) with raw counts as input and the Benjamini-Hochberg procedure was used to estimate FDR. Genes were considered as significantly differentially expressed if P-value<0.05 and log2FC>|0.4| (mouse data) or P-adj<0.05 and log2FC>|2| (clinical data). Gene ontology/biological function was performed with DAVID bioinformatics website.

3.9.3 16S rRNA-sequencing

DNA was isolated from stool samples from WT and ERβKO^{Vil} mice of both sexes. Metagenomic studies are commonly performed on the 16S rRNA gene, which is approximately 1,500 bp in size and contain nine variable regions, located between several conserved regions. The variable regions are frequently used for phylogenetic classification. Library preparation is done in a two-step PCR process, where the variable V3-V4 genomic regions of bacterial 16S rRNA gene is first amplified in the primary PCR reaction. Next, Illumina adaptors are incorporation in the second PCR reaction. DNA was then denatured into single-stranded DNA that was sequenced as described above. An average of 7000 reads per sample were generated. The SILVA database was used for taxonomic assignment and changes in the microbiota composition were analyzed by rarefaction curves, alpha-diversity, Bray-Curtis divergence, and linear discriminant analysis (LDA) effect size (LEfSe). Permutational multivariate analysis of variance (PERMANOVA) was used for statistical analysis. Enriched features were determined with LEfSe, with a LDA threshold of 2.0 and a significance cutoff less than 0.05. Finally, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software was used to predict the functionality of the metagenomic content, based on the abundance of corresponding operational taxonomic units (OTUs). The limitation with this study is the use of stool samples as a proxy for the gut microbiota, which is significantly different from the mucosa microbiota. Moreover, the microbiota changes along the GI tract due to differences in the environment, including pH, transit time, nutrient sources, mucin and bile acids, which again highlights the complexity in using stool as a proxy for the mucosal bacteria. However, due to the non-invasive sampling of stool it is easily translated into the clinic and therefore most of the gut microbiota research has been carried out in stool.

3.10 FEATURE SELECTION METHODS

CRC clinical samples were used for biomarker discovery. Feature selection was used to reduce the high dimension and noise produced by transcriptomic analysis to improve the identification of relevant CRC-related genes. FPKM values were used as input for feature selection using the Vita package in R (threshold for pvalues of 0). The features selected with

Vita were then either combined with the Boruta package or the mRMR package in R, with threshold for pvalues<0.01. Vita is a tree-based method that randomly splits the dataset into two equally sized subsets, and two random forests (RF) are trained using one of the subsets. Feature importance is then estimated based on the other, independent subset and the final importance value is calculated by an average of the two estimated scores for each variable. P-values can be calculated based on the resulting empirical distribution. Boruta is a wrapper method built around the RF classification algorithm. It creates shadow features by replicating and randomly shuffles the original data. This shadow dataset is attached to the original data and then it trains a classifier on the dataset, i.e. RF. A feature is considered to be important if the importance score of the original data is higher than the highest importance score recorded among the shadow features. mRMR is a filter method that select features with a high correlation with the output (i.e. relevance), and a low correlation between themselves (i.e. redundancy). The features are selected one by one by maximizing the objective functions, a function of relevance and redundancy.

3.11 MACHINE LEARNING

The output from the feature selection was used as input for machine learning in order to rank the features based on importance. RF or adaptive boosting (AdaBoost) was used for classification modeling, to keep consistency with tree-based feature selection algorithms. The data was split so that one third was used to train the model and two third was used for testing the model, and the number of estimators used was set to 100.

RF is a tree-based supervised learning model that splits the data into several decision trees, trained on different parts of the same training data and combined to improve the performance. In other words, several weak classifiers are put together to form a strong classifier. Each tree is built based on random extraction of observations and features from the dataset. Each tree is also based on a sequence of yes and no questions, which is based on single or combinations of features. At each node, a question is asked and the tree divides the data into two new branches (new nodes). This is repeated until you reach a leaf, i.e. when the node does not have any branches and the classifier successfully have split the data. The goal is to end up with features that split the observations so that the resulting groups are as different from one another as possible and that the members of each subgroup are as similar as possible.

AdaBoost is a boosting algorithm, which uses an iterative approach to correct the errors from the weak classifiers to turn them into strong classifiers. Like RF, AdaBoost uses decision trees, but these trees consist of only one node and two leaves. AdaBoost uses a forest of such stumps rather than trees and can only use one variable to make a decision. The next tree is trained to take the previous tree´s mistakes into account and focuses on correctly classify the previously misclassified sample. It assigns higher weight to wrongly classified observations while putting less weight on those already classified well. This process is iterated until the data fits without errors or until it has reached the specified number of maximum estimators.

Synthetic minority oversampling technique (SMOTE) or randomly oversampling was used on the imbalanced TCGA data before classification. SMOTE works by drawing lines between existing minority samples in space, and creating new minority samples randomly along the lines. Randomly oversampling is based on randomly duplicating minority samples. Four different combinations were used and the accuracy, precision, recall and AUC were recorded for each combination.

3.12 SURVIVAL ANALYSIS

Scaled and mean-centered FPKM values together with living days were used as input for the survival analysis. High expression and low expression were defined as scale and mean-centered FPKM values above zero and below zero, respectively. Kaplan-Meier curves were plotted in python and the significance was tested with log-rank test.

3.13 STATISTICAL ANALYSIS

GraphPad Prism was used for statistical analysis (GraphPad Software Inc). The results are presented as mean ±SEM. A (two-tailed) Welch's t-test or paired t-test (if paired data) was used for comparison between two groups. One-way and two-way analysis of variance (ANOVA) was used for comparison between multiple conditions followed by fisher's LSD test (*in vivo* data) or Tukey's multiple comparisons test. A p value <0.05 was considered being statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). # Indicates significant sex differences.

4 RESULTS AND DISCUSSION

This section briefly summarizes the main findings of the four papers I-IV, which is a combined effort to dissect the role of estrogen signaling during colon inflammation and CRC. Detailed information can be found in the papers.

4.1 PAPER I: INTESTINAL ESTROGEN RECEPTOR BETA SUPPRESSES COLON INFLAMMATION AND TUMORIGENESIS IN BOTH SEXES

Several studies support a role for ERβ in CRC-preventative effects. However, its general expression and functions are controversial due to relative low mRNA levels in the colon and the use of unspecific antibodies in the literature. Further, support of the impact of ERβ on colitis and CA-CRC has been generated using βERKO mice or ERβ-selective agonists, hence it is unknown through which cells ERβ mediates its protective effects. ERβ is for example also expressed in immune cells, including B cells, T cells, and NK cells [203]. Whether or how intestinal epithelial ERβ is protective against colitis and CA-CRC has not been examined and delineating this mechanism is crucial since selective activation of ERβ can provide novel opportunities for CRC chemoprevention. In order to define the role of intestinal epithelial ERβ during CA-CRC we utilized mice lacking ERβ specifically in the intestinal epithelial cells ($ERβKO^{Vil}$) and induced colitis (9 weeks treatment) and CA-CRC (15-16 weeks treatment) using AOM/DSS.

First, we demonstrated ERβ expression in human colonic epithelial cells and lack of expression in CRC using a validated ERβ antibody, which reveals the clinical relevance to study the role of ERβ as a chemopreventive target against CA-CRC. Next, we showed that ERβ acts protective against AOM/DSS-induced tumor formation in mice, in a sex-dependent manner (Figure 8). $ERβKO^{Vil}$ male mice developed significantly more tumors compared to its WT counterpart, whereas $ERβKO^{Vil}$ female mice presented significantly larger tumors compared to WT females. Moreover, $ERβKO^{Vil}$ mice showed a significant increase of TNFα and NFκB target genes, especially in males. Whereas $ERβKO^{Vil}$ females presented increased ulcerated areas and delayed ulcer healing, which may be explained by the impaired epithelial proliferation required for intestinal regeneration. Furthermore, we showed that intestinal epithelial ERβ could suppress TNFα-induced signaling *in vitro* using human CRC cell lines and protect against TNFα-induced epithelial cell damage *ex vivo* in mouse intestinal organoids. We could thus dissociate the effect from the systemic immune response and the microbiota and determined a local interaction between intestinal epithelial ERβ and TNFα-signaling. In fact, ERβ counteracted the TNFα-induced NFκB signaling both *in vitro*, in human CRC cell lines, and *in vivo*. Our mechanistic experiments revealed that while ERβ did not impact the transactivation of TNFα or the p65 nuclear translocation, TNFα enhanced the transactivation of ERβ. Interestingly, our chromatin immunoprecipitation (ChIP)-qPCR experiments showed a mechanism whereby ERβ directly binds to cis-regulatory chromatin region of key NFκB regulators. ERβ enhanced the expression of activating transcription factor 3 (ATF3), a negative regulator of NFκB, by binding to an assumed enhancer. ERβ further attenuated the positive regulators of NFκB, including B-cell lymphoma 3-encoded

protein (BCL3), and baculoviral IAP repeat containing 3 (BIRC3) by binding close to the promoter. We thus suggest that ERβ attenuates TNFα-induced effects by direct suppression of NFκB-induced inflammatory genes, including monocyte chemoattractant protein-1 (MCP-1/CCL2) and macrophage inflammatory protein-1β (MIP-1β/CCL4), through binding and modulation of NFκB regulators. CCL2 and CCL4 are chemoattractants of MΦ and T cells, and intestinal epithelial ERβ may thus impact recruitment of immune cells, which in turn can impact colitis and CA-CRC.

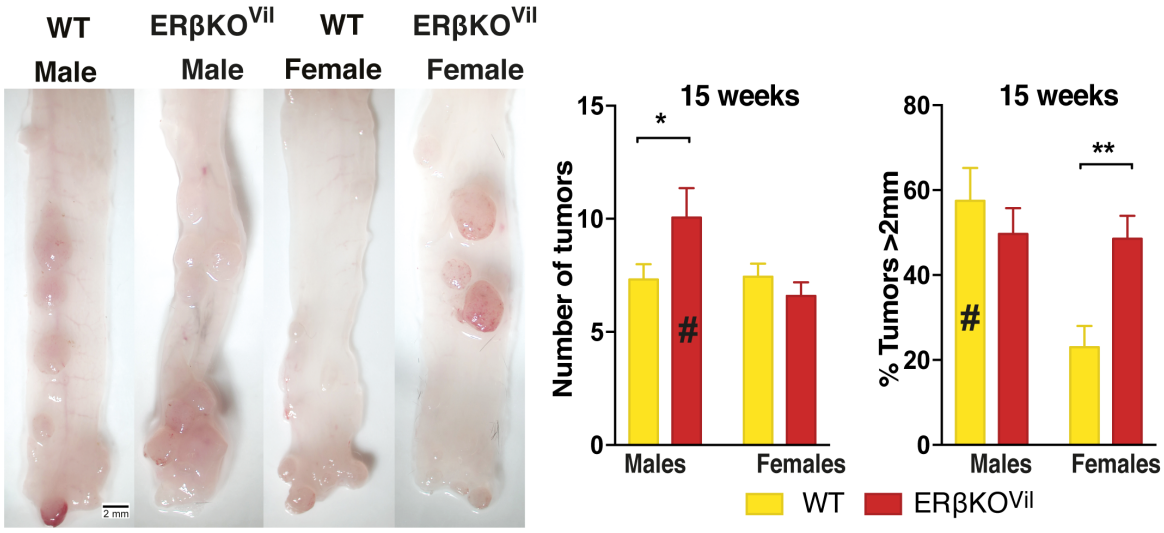

Figure 8: Intestinal epithelial ERβ protects against AOM/DSS-induced tumor formation in both sexes, by reducing the number of tumors in males and the size of the tumors in females. A p value <0.05 was considered statistically significant (* p<0.05, ** p<0.01). # Indicates significant sex differences.

In conclusion, our results show for the first time that intestinal epithelial ERβ protects against CA-CRC in both sexes and determine a possible mechanism, involving an intricate crosstalk between intestinal epithelial ERβ and TNFα-induced NFκB signaling (Figure 9). TNFα enhances ERβ activation and ERβ, in turn, represses the NFκB-signaling by binding and regulating NFκB modulators. The subsequent reduced inflammatory signaling is manifested by reduced secretion of the chemoattractants CCL2 and CCL4, which in turn can cause less recruitment of TNFα-secreting pro-inflammatory MΦ. We thus suggest an inhibitory feedback mechanism where the protection is mediated in a dual manner: 1) ERβ reduces the intrinsic inflammatory signaling in the colon itself, which leads to 2) reduced immune cell activity, and less immune cell-secretion of TNFα.

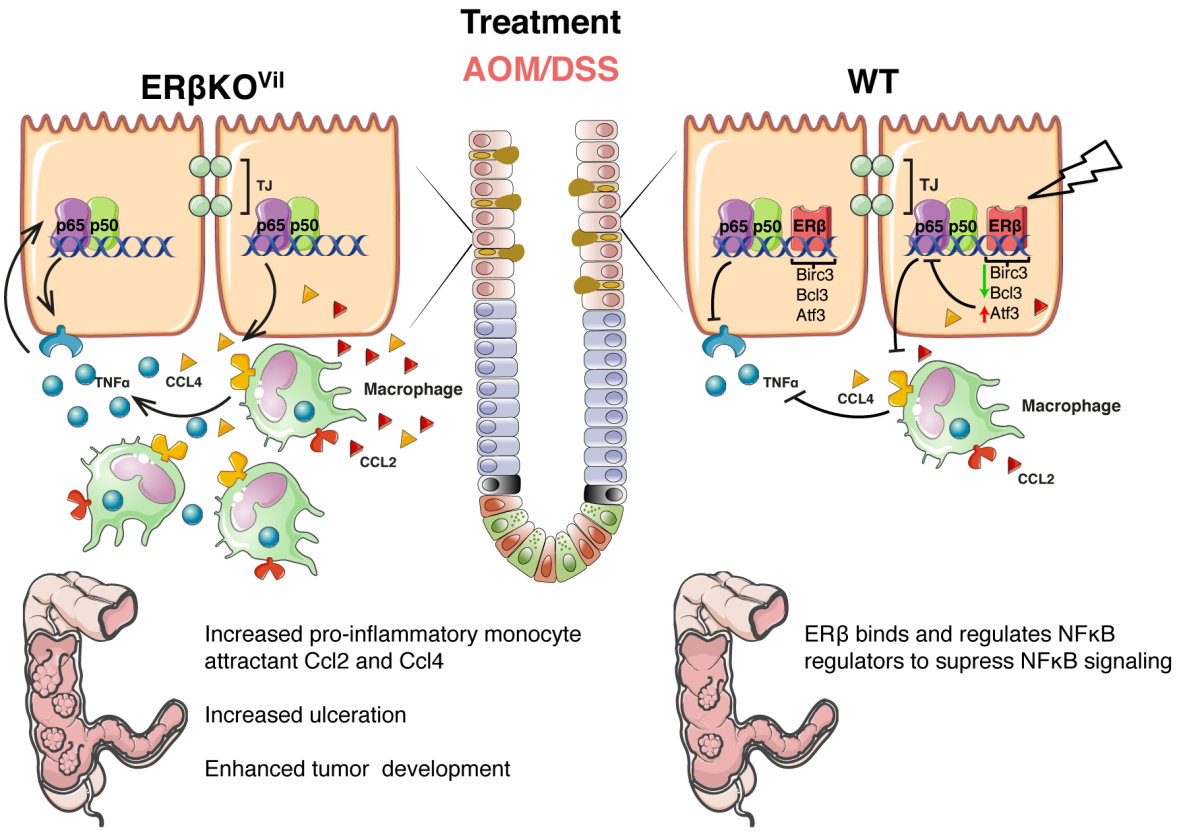

Figure 9: Schematic illustration of the proposed model for ERβ attenuated CRC development. Intestinal epithelial ERβ reduces TNFα signaling through direct mediated regulation of NFκB regulators, leading to suppression of NFκB-induced inflammatory signaling, including less secretion of CCL2 and CCL4, which can lead to reduced recruitment of pro-inflammatory TNFα-secreting macrophages and reduced tumor formation. Note: this image contains elements that were modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License.

4.2 PAPER II: COLITIS-INDUCED COLORECTAL CANCER AND INTESTINAL EPITHELIAL ESTROGEN RECEPTOR BETA IMPACT GUT MICROBIOTA DIVERSITY

As mentioned in **paper I**, there are several studies that support a protective role of ERβ in the pathogenesis of CA-CRC. In the previous paper we showed that intestinal epithelial ERβ protects against CA-CRC in both sexes, in a sex-dependent manner, through an intricate crosstalk with ERβ and TNFα-induced NFκB signaling. We thus demonstrated that ERβ could modulate the response of TNFα regardless of the microbiota composition and immune response *in vitro* and *ex vivo*. However there are evidence that both E2 and genistein regulates the gut microbiota [187 186 195] and intestinal epithelial ERβ may modulate the gut microbiota during colitis and CA-CRC, which could contribute to the enhanced progression of CA-CRC seen in the ERβKO^{Vil} mice. In order to characterize the impact of intestinal epithelial ERβ on the microbiota composition during CA-CRC we performed 16S rRNA sequencing on stool samples collected at sacrifice.

First we showed that AOM/DSS treatment significantly reduced the α-diversity (Figure 10A). Further, we noted a significant compositional dissimilarity caused by treatment. The gram-negative bacteria *Akkermansia* (phylum Verrucomicrobia) and *Mucispirillum* involved in mucin degradation and *Parasutarella* (phylum Proteobacteria) were increased during colitis.

Interestingly, a reduction of SCFA-producing bacteria including *Lachnospiraceae* and *Ruminococcaceae* (phylum Firmicutes) was also seen. Functional prediction of the altered microbiota upon treatment revealed pathways involved in immune system disease and metabolism, including starch and sucrose metabolism. Furthermore, since ERβKO^{Vil} mice showed a sex-dependent increase of colitis-induced tumor formation in **paper I**, sex differences in the microbiota composition might contribute to this. Interestingly, we noted a significant compositional dissimilarity between sexes at baseline, which disappeared with treatment. Females showed significant higher levels of *Bifidobacterium*, which have been associated with health benefits in the intestinal tract and is widely used as probiotics [204]. The probiotic *Bifidobacterium animalis* subsp. *lactis* strain BB12 protects against DSS-induced colitis by reducing TNFα-mediated intestinal epithelial cell apoptosis [205]. Functional prediction showed that the female enriched microbiota impacted metabolism of cofactors and vitamins and the endocrine system. In males we found an increase of the mucus-degrading bacteria *Mucispirillum* and several SCFA-producing bacteria. Worth noting is that the significant increase of pathogenic *Parasutarella* upon AOM/DSS treatment were specific for males, and *Parasutarella* is known to increase in patients with Crohn's disease. Both *Mucispirillum* and *Parasutarella* can secrete LPS, which can impact colitis and CA-CRC through TLR signaling. Studies have shown that males present increased LPS-related functional pathways compared to females, which are reduced upon E2 treatment during Western diet consumption [195]. These microbiota-related sex differences might contribute to the sex differences noted in colitis-induced CRC in **paper I**.

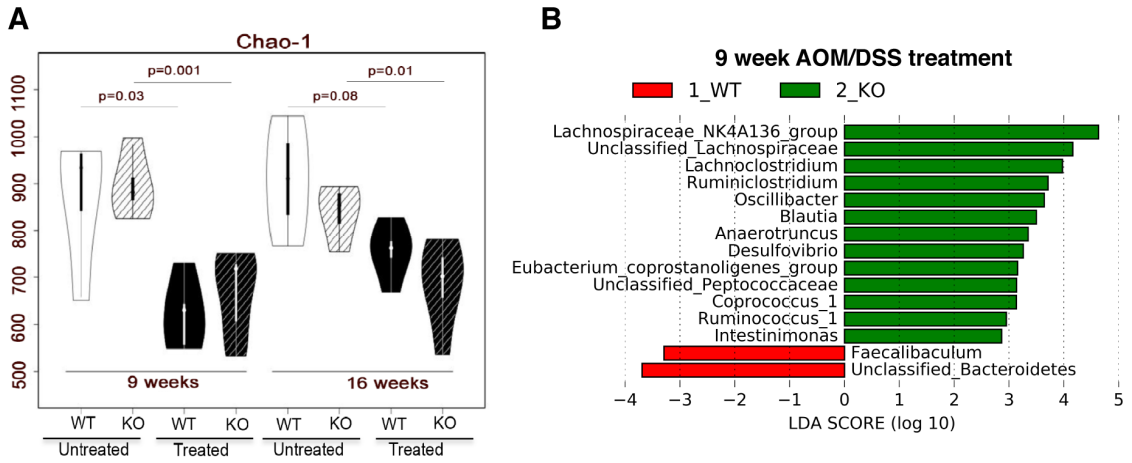

Figure 10: (A) AOM/DSS treatment and loss of intestinal epithelial ERβ reduces the α-diversity during 16weeks treatment. (B) Intestinal epithelial ERβ presents significantly altered taxa abundance during 9weeks treatment, including increased abundance of the sulfate reducing bacteria *Desulfovibrio*.

Interestingly, we noted a significant compositional dissimilarity in ERβKO^{Vil} mice compared to WT mice during colitis. This difference decreased during CA-CRC, but ERβKO^{Vil} mice presented the lowest α-diversity during CA-CRC (Figure 10A). ERβKO^{Vil} mice displayed increased levels of many SCFA-producing Firmicutes families and increased levels of the gram-negative major sulfate reducing bacteria (SRB) *Desulfovibrio* (phylum Proteobacteria) during colitis (Figure 10B). Functional prediction revealed that ERβKO^{Vil} mice presented

alterations in energy metabolism, including enhanced carbohydrate metabolism, and decreased bacteria impacting the endocrine system. Increased levels of SCFAs such as butyrate can inhibit intestinal stem cell proliferation [73]. Thus the increased levels seen in ERβKO^{Vil} mice may inhibit stem cell proliferation, and fail to repair the DSS-induced ulcers, which reflects what we observed in the ERβKO^{Vil} female mice in **paper I**, a delayed ulcer healing. Another interesting finding is the noted increase of the SRB *Desulfovibrio* in ERβKO^{Vil} mice. This strain belong to the main producers of hydrogen sulfide (H_2S), which can be detrimental to the colonic epithelium because it impairs DNA repair and inhibits butyrate oxidation [206]. IEC can starve without butyrate oxidation since it represent 70% of the total energy requirement [206], which may contribute to increased intestinal permeability and impaired intestinal homeostasis. The impaired butyrate oxidation in ERβKO^{Vil} mice may lead to SCFAs accumulation, inhibition of stem cell proliferation, and thereby contribute to the impaired ulcer healing seen in ERβKO^{Vil} female mice in **paper I**. In addition, H_2S can reduce disulfide bonds in the mucin, exacerbating mucin degradation [207], and lead to an impaired intestinal barrier. However, we did not measure the levels of SCFAs or H_2S in the stool, and we did not look at the mucosal-associated microbiota or metabolites. Performing such assays would provide an improved understanding of the impact of the gut microbiome on the colon.

In conclusion, we show for the first time that intestinal epithelial ERβ modulates the gut microbiota, including bacteria involved in metabolism, during AOM/DSS-induced colitis, which may impact intestinal homeostasis and contribute to the CA-CRC progression.

4.3 PAPER III: HIGH-FAT DIET AND ESTROGEN IMPACT THE COLON AND ITS TRANSCRIPTOME IN A SEX-DEPENDENT MANNER

In **paper I** and **II** we showed that intestinal epithelial ERβ protected against colitis and subsequent tumor formation in both sexes. A major risk factor for CRC is obesity and consumption of a Western diet, and the incidence of CRC is increasing in young adults. Colon has been identified as the first organ to respond to HFD, with increased inflammatory signaling, intestinal permeability, stem cell activity and altered gut microbiota [45-53], which could contribute to CRC. Obesity is a risk factor for CRC in both sexes, however, this association is stronger in men compared to women [208], which indicates a role for estrogen. Understanding the impact of estrogen signaling during HFD-induced colon inflammation may improve our understanding of ERβ as a preventative target against CA-CRC. In order to dissect this, mice of both sexes were fed a HFD (60% fat) or a matched control diet (CD, 10% fat) for 13 weeks and treated with different ER-selective ligands for the last three weeks prior to sacrifice.

First we demonstrated major sex differences in the colon transcriptome, especially during CD. The genes responsible for the sex differences during CD belonged to pathways involved in immune response and cell proliferation. Less distinct sex differences were seen during HFD, but common for both dietary conditions were sex differences involved in circadian/rhythmic processes, including the core clock genes *Npas2* and *Arntl (Bmal1)*, which were higher expressed in females compared to males. As we identified large sex differences

during CD it was not a surprising finding that the sexes responded differently to HFD in terms of colonic gene expression, with less than 10% in common. Interestingly, HFD impacted circadian clock genes, genes involved in oxidation-reduction and inflammatory responses in both sexes. Some of the sex-specific genes belonged to common pathways, such as cell proliferation, cell adhesion, migration, angiogenesis, and immune system, whereas some genes were involved in pathways tied to sex. Cell cycle, hypoxia and glucose homeostasis were specifically enriched in males, whereas Wnt signaling, apoptosis, lipid metabolism, and steroid hormone signaling were enriched in females. Thus, we showed for the first time that sex impacts the transcriptomic response to HFD in colon and we further demonstrated that this manifested in functional changes in the colon. Both sexes showed a significant increase of macrophage infiltration upon HFD, with a stronger increase in females. Only males presented a significantly increased epithelial cell proliferation upon HFD.

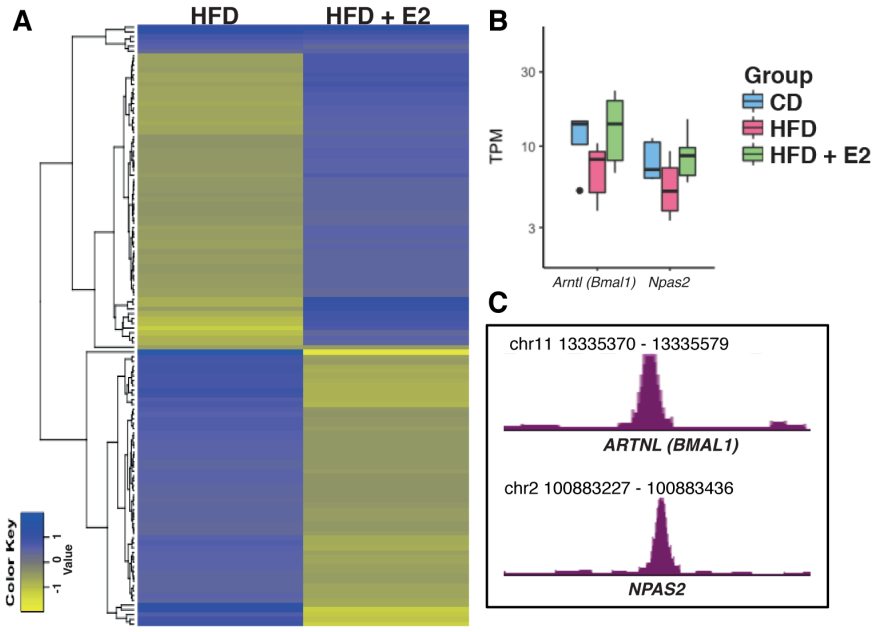

Figure 11: E2 regulates a fraction of HFD-induced genes, interestingly almost all of these HFD regulated genes were regulated in the opposite direction upon E2 treatment in males. The circadian core clock genes *Bmal1* and *Npas2* were downregulated by HFD and the levels were brought back to normal with E2 treatment. ChIP-seq in human CRC cell lines revealed that both *ARNTL* and *NPAS2* had ERβ DNA-binding sites, which suggests ERβ binding and regulation of these genes.

Interestingly, E2 treatment in males affected expression of 30% of the HFD-deregulated genes, of which almost all were regulated in the opposite direction upon E2 treatment (Figure 11A). These genes were involved in pathways related to cell cycle and rhythmic processes. E2 opposed the HFD-induced downregulation of both *Npas2* and *Bmal1* (Figure 11B). The circadian clock regulates vital processes involved in colitis and CRC including immune response [209], intestinal permeability [210] and cell cycle control [211]. BMAL1 has been shown to repress chemokines associated with recruitment of pro-inflammatory MΦ [212]. Interestingly, we found a decreased MΦ infiltration with E2 treatment, which also was evident with ERβ-selective activation with DPN in both sexes. Moreover, E2 treatment and ERβ-selective

ligand activation in males reduced the HFD-induced epithelial cell proliferation. Our mechanistic data in human CRC cell lines showed that ERβ could bind to cis-regulatory chromatin regions of both *BMAL1* and *NPAS2* (Figure 11C), which suggest that ERβ could act locally in the colon to directly regulate these genes. We confirmed this using our ERβKO^{Vil} mice, where knockout females showed a significant decrease in *Bmal1* and *Npas2* during HFD.

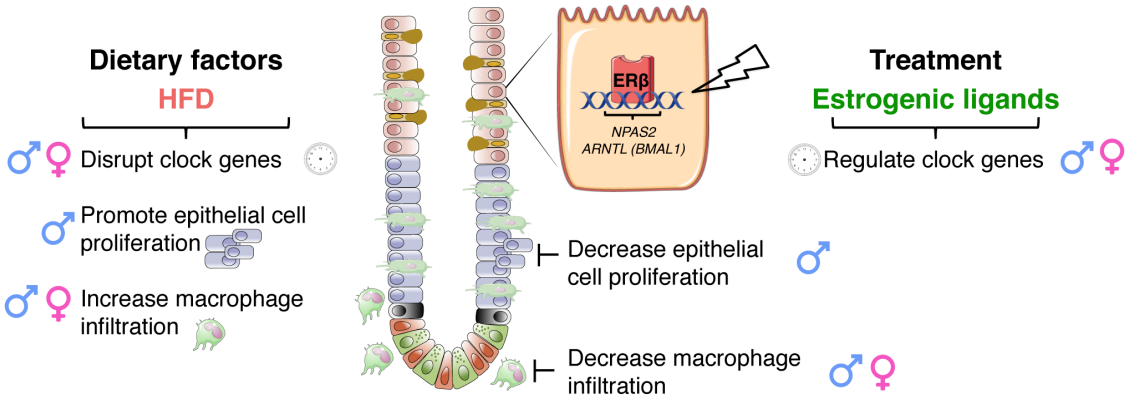

Figure 12: Schematic illustration of the proposed model for estrogen regulation, via ERβ, on the colon microenvironment during HFD-induced obesity. Estrogen signaling, via ERβ could modulate the HFD-induced epithelial cell proliferation, macrophage infiltration and regulation of core clock gene expression, in a sex-dependent manner. Note: this image contains elements that were modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License.

In conclusion or study show for the first time that estrogen-signaling via ERβ can modulate the colon microenvironment during HFD, including regulation of the core clock genes *Bmal1* and *Npas2*, epithelial cell proliferation and MΦ infiltration, in a sex-dependent manner (Figure 12). This study provides new insights how ERβ protects against detrimental effects of HFD-induced colon inflammation, and potentially undermines the promotion of CA-CRC.

4.4 PAPER IV: THE IMPORTANCE OF SEX IN COLORECTAL CANCER BIOMARKER DISCOVERY

In addition to better CRC preventatives, there is a need for better diagnostic and prognostic biomarkers, which could significantly improve the CRC survival rates. Despite the intense research, few biomarkers have reached the clinic. The use of gene expression data for biomarker discovery has been limited. Gene expression data is usually obtained from small sample sizes but with high dimensions and noise, which usually leads to overfitting problems. The advances in both omics technologies and bioinformatics have the possibility to improve and accelerate current strategies for biomarker discovery. Feature selection in combination with machine learning reduces the noise and the high dimension produced by transcriptomic analysis, which will help to identify relevant disease-related genes. However, there is a need to account for sex, which studies fail to do. CRC presents sex differences in the incidence, prognosis, mortality and tumor characteristics. In this study we looked at transcriptomic sex differences in paired normal and matched CRC samples (n=24, 18 women and 6 men) and in

641 (299 women and 342 men) CRC (COAD and READ) and 51 (28 women and 23 men) noncancerous mucosal tissues from TCGA data. We performed feature selection with Vita, Boruta and MRMR, and machine learning using random forest to identify sex-specific diagnostic biomarkers. Further, we used the TCGA data to perform sex-specific survival analysis to identify their prognostic value.

Interestingly, we found sex differences in the normal colon and CRC transcriptome. The differentially expressed genes in the normal colon belonged to pathways involved in bile acid secretion, IBD, epithelial cell differentiation, PPAR signaling and carbohydrate-, lipid-, and vitamin metabolism. Sex differences in CRC transcriptome were indicative of differences in proliferation, immune response and B-cell receptor signaling. Furthermore, our data-driven biomarker discovery revealed both common and sex-specific top-ranked biomarkers. Biomarkers common between the sexes included previously proposed diagnostic CRC biomarkers, such as *CLDN1, CEMIP* and *CDH3* [213]. Interestingly, we also found sex-specific top biomarkers (Figure 13). This included *ETV4, FOXQ1, INHBA* and *ESM1* in females and *TRIB3* in males, all of which previously have been reported to be deregulated in CRC. Moreover, we also found significant sex-specific prognostic value of previously proposed prognostic biomarkers, including *CLDN1* and *ESM1*. *CLDN1* and *ESM1* were upregulated in CRC and *CLDN1* showed an unfavorable prognostic value specifically in females, whereas *ESM1*, showed an unfavorable prognostic value specifically in males. Finally, we proposed some entirely novel diagnostic and prognostic biomarkers, including *TSPAN7* (females, unfavorable), *SLC25A23* (females, unfavorable) and *C2orf88* (males, favorable).

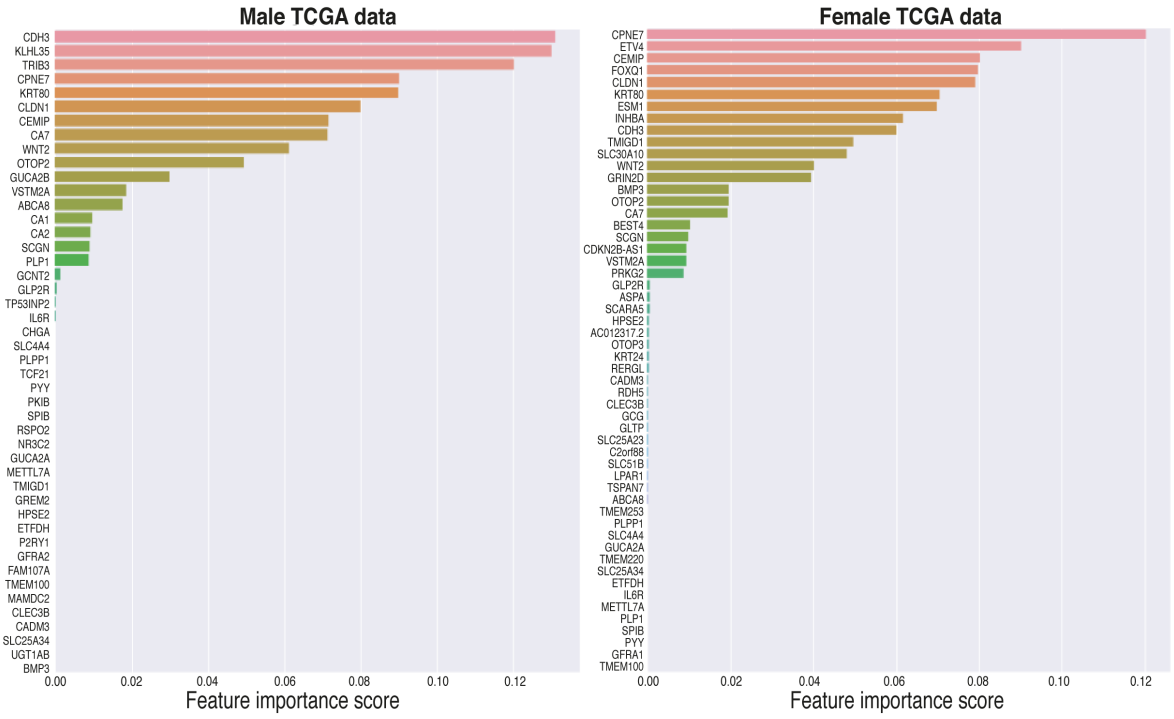

Figure 13: Feature importance ranking for males and females reveals common and sex-specific top diagnostic biomarkers. Feature selection is based on Vita, Boruta and mRMR and importance ranking are based on machine learning with random forest on COADREAD TCGA data.

In conclusion, we identify sex differences in the normal colon and CRC transcriptome, which may help explain the sex differences in CRC susceptibility and mortality rates. Interestingly, we also found sex-dependent diagnostic and prognostic biomarkers candidates, and we propose that including sex considerations and machine learning can improve the discovery of diagnostic and predictive biomarkers. This can help improve personalized medicine and CRC survival.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work presented in this thesis primarily relates to sex differences and the role of intestinal epithelial ERβ during colitis and colitis-induced CRC. This section further discusses the significance of the findings and future directions.

CRC is the third most deadly forms of cancer in the Western world. Although screening-efforts have reduced the overall mortality, the incidence is increasing among young adults. The frequency of IBD and obesity are increasing in parallel, which suggests a common underlying environmental link between the conditions. This increase is thought to correlate to an increased intake of high fat diets, and obesity is a major risk factor for CRC. The risk-benefit balance of current CRC preventative treatments is poor, and there is a need for safer and better preventatives in order to reduce CRC mortality. There is evidence that ERβ is protective against CRC and natural ERβ-selective agonist has been proven safe in phase II clinical trials [214, 215], however the underlying protective mechanism needs to be elucidated. In **paper I** we showed that intestinal epithelial ERβ is protective against colitis and colitis-associated tumor formation in both sexes. Furthermore, in **paper III** estrogen signaling via ERβ could protect against HFD-induced detrimental effects on the colon microenvironment. ERβ-selective activation reduced the HFD-induced epithelial cell proliferation in males and macrophage infiltration in both sexes. This protective effect could, in part, be accompanied by ERβ-induced modulation of the gut microbiota, and we showed that intestinal epithelial ERβ modulated the gut microbiota during experimental colitis in **paper II**.

In **paper I** the underlying protective mechanism by ERβ involved a cross talk with TNFα/NFκB signaling and a proposed direct ERβ-mediated repression of NFκB activators, resulting in reduced inflammatory signaling and tumor formation. This was accompanied by an altered abundance of SCFAs- and H_2S-producing bacteria by ERβ, as shown in **paper II**. SCFAs and H_2S are bacterial metabolites that present potential detrimental or beneficial effects for the intestinal epithelium. In **paper III**, we showed that ERβ activation reduced the HFD-induced deregulation of core clock gene expression. The circadian clock is important to keep intestinal homeostasis, and disruption can modulate proliferation, intestinal permeability, the microbiota composition and the immune system [210, 216-219].

Interestingly, the circadian clock has also been shown to be dysregulated in mice with DSS-induced experimental colitis, which suggests a common underlying disease mechanism with HFD-induced colon inflammation. In fact, *Bmal1* knockout mice present increased severity of DSS-induced colitis [220]. Furthermore, myeloid cell-specific deletion of *Bmal1* results in increased expression of *Ccl2* in blood monocytes and increased serum levels during HFD-induced obesity [212]. This increased expression can recruit pro-inflammatory macrophages. In **paper I**, we did see an increase of *Ccl2* upon loss of intestinal epithelial ERβ during colitis-induced CRC in males, which may contribute to an altered tumor

microenvironment and enhanced tumor formation. Since we showed that the *Bmal1* gene has a cis-chromatin ERβ-binding site, it is possible that ERβ directly regulates the intestinal circadian clock during colitis and thereby attenuates AOM/DSS-induced tumor formation. The gut microbiota and the TLR expression are also under circadian regulation, and disruption of these rhythms can result in regulation of bacterial metabolites that can influence the intestinal homeostasis.

Another important finding, are the significant sex differences seen in all papers. In **paper I**, intestinal epithelial ERβ protected against colitis-induced tumor formation in a sex-dependent manner. ERβKO^{Vil} male mice developed significantly more tumors whereas ERβKO^{Vil} female mice developed significantly larger tumors compared to its WT counterparts. Furthermore, ERβKO^{Vil} females presented more ulcerated areas and a delayed ulcer healing, whereas ERβKO^{Vil} males presented increased expression of several NFκB-induced genes. Unexpectedly, male mice responded stronger to the loss of intestinal epithelial ERβ compared to females, both in terms of inflammatory signaling and tumor development. Estrogen is not though as a male hormone, however E1 and DHEA, a precursor of 3β-Adiol and a proposed ERβ ligand is present in males. Furthermore, the mice were given normal chow, which contains phytoestrogens that activate ERβ. Thus, ERβ would be active in both sexes. Interestingly, in **paper III** the CD and HFD did not contain any phytoestrogens and we noted significant sex differences in the colon transcriptome during both dietary conditions (especially during CD), and in the response to HFD. Furthermore, we observed sex differences in the microbiome, which may promote or prevent the response to experimentally-induced colitis. Interestingly, sex differences were also seen in the human normal colonic and CRC transcriptome in **paper IV**. The sex differences of the normal colon were overrepresented among pathways related to bile acid secretion, IBD, epithelial cell differentiation, PPAR signaling and carbohydrate-, lipid-, and vitamin metabolism, all known to be dysregulated in CRC. We also noted sex differences in the CRC transcriptome, mostly related to immune response and B-cell receptor signaling. Although ERβ expression is lost in CRC, we still noted sex differences in the CRC transcriptome as well as among proposed diagnostic and prognostic biomarkers. The sex differences may be mediated by ERβ activity in surrounding normal epithelium or in the immune system, or be unrelated to ERβ. In **paper I-III** we showed that epithelial ERβ could modulate the microbiota composition and inflammatory signaling, which in turn could shape the tumor microenvironment, and thereby impact tumor gene expression.

Another interesting finding in **paper I** is the enhanced ERβ transactivation by TNFα. ERβ is expressed at low levels in the intestinal epithelial cells, and it has been unclear if this low expression has a functional role. Here we propose that the inflammatory state amplified the activity of ERβ, which in turn could suppresses the colon inflammation and tumor development induced by HFD and AOM/DSS. All together our results support the notion that an ERβ agonist may constitute a suitable chemopreventive approach against CA-CRC.

5.1 FUTURE PERSPECTIVES

The transactivation of ERβ was enhanced by TNFα suggesting that the inflammatory state could enhance the protective function of ERβ. In addition to TNFα, LPS from gram-negative bacteria can activate NFκB signaling and it is possible that the LPS-induced inflammatory signaling also can enhance transactivation of ERβ. In this case, ERβ in turn, could repress the LPS-induced inflammatory response. Interestingly, colonic ERβ expression is reduced in antibiotic-treated mice [221], suggesting that the gut microbiome and its metabolites or the overproduction of glucocorticoids in microbiota deficient mice might regulate ERβ expression. Characterization of the crosstalk with intestinal epithelial ERβ and LPS-induced inflammatory signaling will improve the understanding of the ERβ-mediated protective mechanism via crosstalk with NFκB. Further studies investigating how ERβ and sex impacts the p65 cistrome in the colon of AOM/DSS treated mice can lead to improved understanding of the crosstalk between ERβ and NFκB. The protective mechanism of ERβ during AOM/DSS-induced tumor formation can further be investigated by transcriptomic profiling of the colon from WT and ERβKO^{Vil} mice.

Several NRs have been associated with colon inflammation and an increasing number of NRs have been validated as potential drug targets for IBD. An improved understanding and identification of novel crosstalk mechanisms can ultimately change the field from a single targeting approach to a dual NR targeting approach. Alternative targeting strategies, such as allosteric ligands and ligands targeting NR related protein-protein interactions, may improve current therapeutic strategies. The BA receptor FXR regulates BA homeostasis and its expression in the intestine is associated with anti-inflammatory effects [85]. Interestingly, sex has been reported to affect BA pool composition and size [222], which indicate a role for estrogen in the regulation of the BA homeostasis. Interestingly, FXR activation has been shown to suppress the expression of the estrogen sulfotransferase (SULT1E), which is responsible for metabolic estrogen inactivation [223]. Moreover, ERα and glucocorticoid receptor (GR) has been shown to crosstalk and to regulate each other's chromatin binding in breast cancer cell lines [224]. In addition to the direct crosstalk, GR can also indirectly affect ERα response through regulation of SULT1E [225]. Characterization of a possible crosstalk between ERβ, FXR, GR, and other NRs in the colon may lead to improved therapeutic strategies.

Furthermore, intestinal epithelial ERβ modulates the gut microbiota and probiotics are promising in the prevention and treatment of CRC. However to further characterize the impact of ERβ on the microbiome, larger studies with metagenomics profiling is needed to improve the taxonomic resolution and to get microbiome functional profiles.

Moreover, we found that intestinal epithelial ERβ regulated the circadian clock gene expression, which is important to keep intestinal homeostasis. The circadian clock is dysregulated in both IBD and CRC. Recent studies have shown that clock gene disruption is an early event in IBD, since clock gene expression was found to be significantly lower even in non-inflamed mucosa of patients with IBD compared to controls [226]. Restoring the

circadian clock by safe therapies, such as selective activation of ERβ, can be an ideal approach for IBD and in the prevention of CA-CRC. However, further studies are needed to characterize the role of ERβ in the regulation of the circadian rhythmicity in the colon. Studying mice at different zeitgeber times are thus crucial in order to understand how intestinal ERβ regulates the impaired circadian rhythmicity during HFD and experimentally-induced colitis.

Worth noting is that some of the sex differences reported throughout the thesis were related to immune cell response, including B-cell receptor signaling (both in mouse and humans). Although estrogen and ERβ is known to impact the immune system, the vast majority of immune-related genes are located on chromosome X and skewed X chromosome inactivation can lead to sex-biases in inflammatory diseases. In order to detail estrogen related sex differences, a larger study including normal colon and CRC from pre- and postmenopausal women would be needed. However, ERβ is also expressed in various immune cells and may impact the immune response. To characterize the role of ERβ expression in immune cells T or B cell-specific deletion of ERβ can be utilized.

After characterization of the mechanism, the next step would be to screen for a safe ERβ-selective agonist and test it in clinical trials to see if it can reduce the inflammation in patients with IBD and to prevent the development of CA-CRC.

6 ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to all the people that have been involved in the work presented in this thesis, without you this would not have been possible.

First, I would like to thank the funding that supported this work, including the National Cancer Institute at the National Institutes of Health, the Swedish Cancer Society, the Swedish Research Council and the Stockholm County Council.

I especially would like to thank my main supervisor **Cecilia Williams** for welcoming me into the lab and giving me the opportunity to be a part of many exciting projects and for being a true inspiration and role model. You have inspired me and guided me to grow as a scientist with your dedicated passion for research and your ability to look at challenges from a positive angle. I always left a meeting with you filled with new energy and inspired to tackle any possible challenges. During my PhD you have always made time to help me and to provide feedback, and for that I will always be grateful.

I would also like to thank my co-supervisor and group member **Amena Archer** for the great support and close collaboration in many projects. You have been my guide in the lab but also guided me into becoming a better scientist by your critical thinking and questions. We have spent some late nights together in the lab, in the animal facility and some nights with pizza to finish manuscripts. Thanks for your constant patience and feedback you have provided during these years and for inspiring me to always be critical and curious. Thanks to my co-supervisor **Anders Andersson** for your support and discussion regarding the microbiota analysis when needed.

I would like to thank present and past group members of the Williams lab who have created a great and inspiring work environment during these years: **Rajitha Indukuri** for your positive energy during all our work together. We registered as PhD students at the same day and you have been my partner in crime during these years. Thanks for all the fun we have had on courses and conferences in Shanghai, Washington DC and Greece. **Madeleine Birgersson** for your dedicated work spent in the lab and for always helping me out with experiments in different projects. **Dandan Song** for your great company and enthusiasm. **Mohammed Hakim Jaffer Ali** and **Ahmed Ibrahim** for good discussions when it comes to method optimizations. **Fahmi Mesmar** for fruitful discussions in the beginning of my thesis and for always making me laugh with your funny jokes. To all master students that contributed to the work.

I would further like to thank all my coauthors and research collaborators: **Rodrigo Lozano** and **Pekka Katajisto** for the help with the organoid work. **Johan Hartman** for providing patient samples. **Christina Savva** for the help with the animal work and for always providing great coffee. **Marion Korach-André** for the help with animal work and for giving valuable input on the metabolic aspects. **Lars Engstrand** and **Luisa Hugerth** for the assistance with 16S rRNA sequencing and analysis.

Thanks to the members of my half-time examination board: **Sam Okret**, **Marco Gerling** and **Carsten Daub** for valuable feedback at my half-time.

Thanks to **Joëlle Rüegg** for being my opponent and **Anita Sjölander**, **Olle Stål** and **Carsten Daub** for taking part of the examination board.

Thanks to my mentor **Claudia Kutter**, fortunately we did not have any issues to discuss. Thanks for your advice in my research projects and for scientific career advice.

Thanks to **Monica Ahlberg** at the KI administration for all your help with my numerous application forms.

I would like to thank the support from the National Genomics Infrastructure (NGI) and SNIC/Uppsala Multidisciplinary Center for Advanced Computational Science for assistance with massively parallel sequencing and access to the UPPMAX computational infrastructure. Additionally, I would like to thank my advisor **Viktor Jonsson** from NBIS/SciLifeLab for input and discussion on RNA-seq analysis and statistics.

Tack till alla mina vänner utanför forsnknigsvärlden för allt stöd och fina stunder.

Slutligen vill jag tacka min kära familj, **Roger**, **Karolina**, **Amanda**, **Lukas**, **Alice**, **Alma**, **Mållgan**, **Milla** och **Mio** för all kärlek och värme och för att ni alltid finns där för mig. **Erik**, tack för din kärlek och ditt enorma stöd och tålamod, och för att jag får dela allt med dig ♥.

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